Atty. Dkt. Nos. 029488-0111 & -0112

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Bruno DE LIGNIERES	Jay BUA
Title:	**TREATMENT OF MASTALGIA WITH 4-HYDROXY TAMOXIFEN**	**REDUCTION OF BREAST DENSITY WITH 4-HYDROXY TAMOXIFEN**
Appl. No.:	10/734,640	10/734,644
Filing Date:	12/15/2003	12/15/2003
Examiner:	U. Ramachandran	B. Fetterolf
Art Unit:	1617	1642
Confirmation Number:	9061	9030

DECLARATION OF DANA HILT, M.D.
UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Dana Hilt, do hereby declare and state as follows:

1. I received my M.D. degree from Tufts University School of Medicine in 1979. Thereafter I trained in Internal Medicine at Harvard Medical School and in Neurology at The Johns Hopkins School of Medicine. I was on the staff of the National Institutes of Health in Bethesda, Maryland from 1983-1988, and then a faculty member of the Neurology and Molecular Biology Departments of the University of Maryland School of Medicine from 1988-1993. In 1993 I joined Amgen to set up their first Clinical Neuroscience group to conduct drug development studies in a variety of medical conditions. Thereafter, I served as the Chief Medical Officer and in senior drug development, medical, and management positions at Guilford Pharmaceuticals, Ascend Therapeutics, Critical Therapeutics, and EnVivo Pharmaceuticals. I am presently Senior Vice President of Development and Chief Medical Officer at EnVivo Pharmaceuticals. I have had drug development experience in a variety of clinical

areas, including neurology, pain, sedation, metabolic/endocrine, oncology, psychiatry, and pulmonary/allergy.

2. From November 2002 through July 2006 I was employed by Ascend Therapeutics, Inc. as Senior Vice President of Development and Chief Medical Officer. I have been retained by Ascend Therapeutics, Inc. as a consultant to provide my professional opinion on the subject matter discussed herein and to prepare this Declaration, for which I am being compensated for my time at my usual consulting rate of $300 per hour.

3. I understand that Ascend Therapeutics, Inc. is the licensee of U.S. Patent Applications 10/734,640 and 10/734,644 ("the applications"), which I have reviewed. I understand that U.S. Patent Application 10/734,640 is directed to treating mastalgia using 4-hydroxy tamoxifen and that U.S. Patent Application 10/734,644 is directed to reducing breast density using 4-hydroxy tamoxifen. I understand that the applications were filed in December of 2003, with priority dates of December of 2002.

4. I have reviewed the Office Action mailed August 23, 2007, in U.S. Patent Application 10/734,640, where the Patent Office Examiner states that the method described in the application is obvious in view of publications including (i) Mauvais-Jarvis, *Curr. Ther. Endocrin. Metab.* 280-84 (1988); (ii) Pujol *et al., Cancer Chemother. Pharmacol.* 36: 493-98 (1995); (iii) Fentiman *et al., Br. J. Surg.* 75: 845-46 (1988); (iv) Mauvais-Jarvis, Senologie & Pathologie Mammaire, 4eme Cong. Int'l, 128-32 (1986); (v) Kochinke, U.S. Patent 5,613,958; and (vi) Malet et al., *Cancer Res.* 48: 7193-99 (1988).

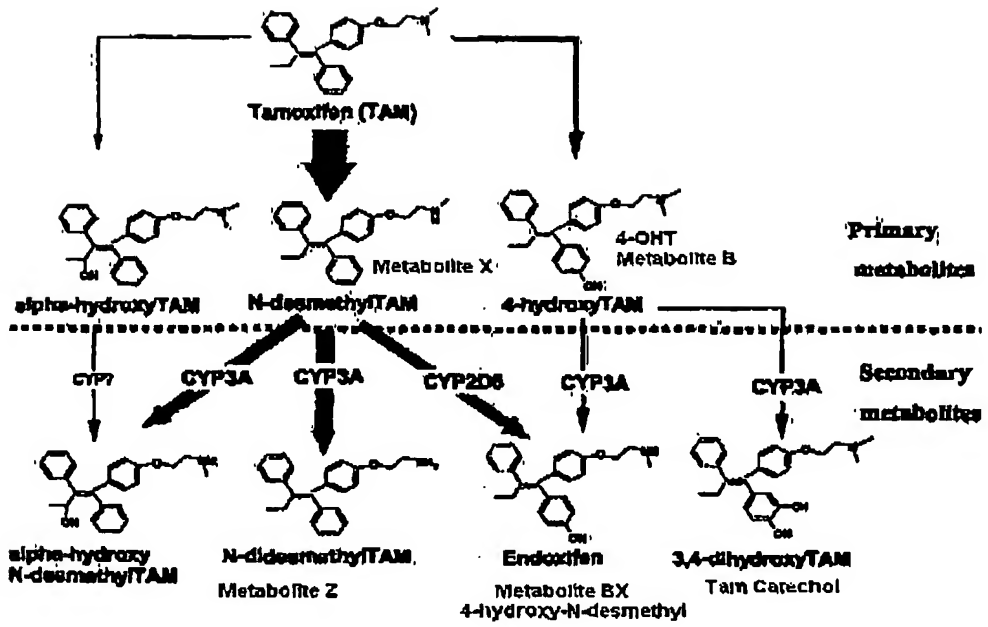
5. I have reviewed the Office Action mailed August 4, 2007, in U.S. Patent Application 10/734,644, where the Patent Office Examiner states that the method described in the application is obvious in view of (i) Atkinson *et al., Cancer Epidem., Biomarkers & Prev. 863-66* (1999); (ii) Boyd *et al., J. Nat'l Cancer Inst.* 87: 670-75 (1995); (iii) Kolb *et al., Radiology* 225: 165-75 (2002); (iv) Mauvais-Jarvis *et al.*, U.S. Patent 4,919,937; (v) Mauvais-Jarvis *et al., Cancer Res.* 46: 1521-25 (1986); (vi) Ueda *et al.*, U.S. Patent 5,045,533; and (vii) Yamaguchi *et al.*, U.S. Patent 5,820,877.

6. I attended the Patent Office interviews conducted on November 14, 2007, when these Office Actions were discussed with Patent Office Examiners.

7. I provide the following statements, which I understand may be used to support the applications. The opinions expressed here are based on my knowledge and experience in the field.

8. I understand the Patent Office Examiners' positions to be based on the following: Certain publications report the use of tamoxifen to treat mastalgia or to reduce breast density, and other publications report that 4-hydroxy tamoxifen (4-OHT) is an active metabolite of tamoxifen, and that 4-OHT can be administered topically. Apparently, the Patent Office Examiners believe that the known status of 4-OHT as an active metabolite of tamoxifen made it obvious to use 4-OHT to treat conditions that have been treated with tamoxifen. I must disagree for a number of reasons.

9. The Examiners assume that an active metabolite can be administered directly instead of the corresponding, clinically proven parent drug, but this assumption overlooks several important pharmacological factors, including some that are particularly relevant to tamoxifen. First, it must he understood that tamoxifen is in no way a "pro-drug" of 4-OHT. Tamoxifen is metabolized into a number of different metabolites, several of which have been shown to be biologically active. The current understanding of the metabolism of tamoxifen is summarized in Desta *et al.*, *Pharm. & Exp. Therapeut.* 310: 1062-75 (2004) (copy attached). The diagram below is an annotated version of Fig. 7 of Desta, showing alternative names for some of the metabolites. As in Desta, the thickness of the arrows indicates the relative contribution of each pathway. This diagram shows that, as currently understood, tamoxifen is metabolized into three primary metabolites, which are further metabolized into four secondary metaholites. 4-OHT is one of the three primary metabolites, and is not even the major primary metabolite.



10. It also is important to understand that *trans (Z)* tamoxifen (the commercially available drug) metabolizes into the *Z* isomer of 4-OHT only. In contrast, when 4-OHT is directly administered, both *Z* and *E* isomers are present in an equilibrium state. If the relevant activity of tamoxifen were associated with only the *Z* isomer 4-OHT metabolite, then administering 4-OHT directly arguably would be expected to result in reduced efficacy, due to a smaller amount of active species being administered. This difference becomes even more significant when the activities of the *Z* and *E* isomers are considered. While the *Z* isomer of 4-OHT bas anti-estrogenic activity, the *E* isomer is a true selective estrogen receptor modulator (SERM) that exhibits both pro- and anti-estrogenic activity. *See, e.g.,* Robertson *et al.*, *J. Steroid Biochem.* 16: 1-13 (1982) (copy attached), at page 8, Table 1 (reporting that the *Z* isomer has a relative binding affinity for the rat uterine estrogen receptor of 285, as compared to only 5 for the *E* isomer). Thus, administering a mixture of the *Z* and *E* isomers of 4-OHT instead of tamoxifen may result in estrogenic activity where only anti-estrogenic activity is desired. Additionally, while tamoxifen has about the same relative binding affinity for the alpba (7) and beta (6) estrogen receptors, 4-OHT has a higher relative binding affinity for the beta estrogen receptor (339) than the alpba estrogen receptor (178). *See, e.g.,* Kuiper *et al.*, *Endocrinol.* 138: 863-66 (1997) (copy attached). Thus,

the use of 4-OHT is in no manner equivalent to the use of tamoxifen alone. At the outset, therefore, the person skilled in the field would have had reasons to question whether 4-OHT could be administered directly, in place of tamoxifen, to achieve the same effect.

11. As noted above, tamoxifen has several active metabolites, many of which were known before the filing date of the applications, as discussed in more detail below. Prior to the work described in the applications, however, there was no scientific basis for predicting that the *Z* isomer of 4-OHT is the relevant tamoxifen metabolite for treating mastalgia or for reducing breast density. Thus, prior to the work described in the applications, persons working in the field could not have reasonably expected to be able to treat mastalgia or reduce breast density with 4-OHT, let alone with a mixture of its *Z* and *E* isomers.

12. Additionally, tamoxifen has many activities other than its anti-estrogen activity. For example, "[t]amoxifen can behave either as a frank estrogen (pure agonist), a partial agonist or as an antagonist, depending on the species, target organs, and end-points assessed." Lonning *et al.*, *Clin. Pharmacokinet.* 22: 327-58 (1992) (copy attached), at 331 (citations omitted). Other proposed mechanisms of tamoxifen action include "inhibit[ing] the conversion of estrone sulfate to estradiol, bind[ing] to 'antiestrogen binding sites,' and inhibit[ing] protein kinase C or calmodulin." *Id.* Prior to the work described in the applications, it was not known that the anti-estrogen activity associated with the 4-OHT metabolite of tamoxifen is a relevant activity for treating mastalgia or for reducing breast density.

13. In summary, because tamoxifen has a number of biologic activities, and a number of biologically active metabolites, *a priori* predicting which specific activity and metabolite of tamoxifen might be useful for the treatment of any breast condition is not an undertaking that can he carried out with any reasonable level of certainty.

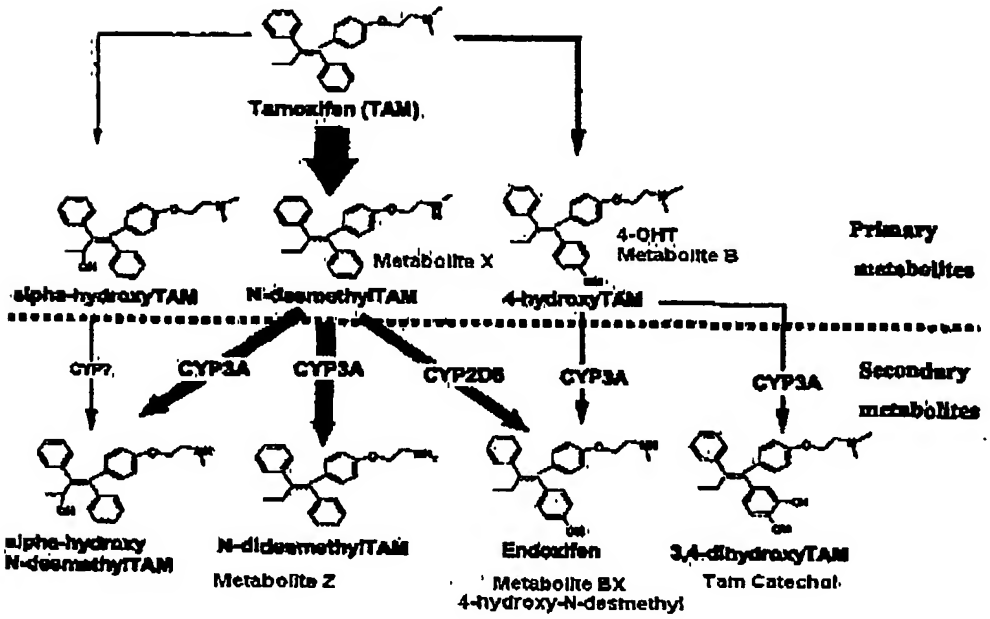
14. Well before the December 2002 priority date of the applications, a number of tamoxifen metabolites had been discovered and studied, several of which had raised interest as pharmacologically active metabolites that might be useful, for example, for

treating breast cancer. For example, Jordan *et al.*, *Breast Cancer Res. & Treat.* 2: 123-38 (1982) (copy attached), presents a review of tamoxifen metabolites, including Metabolite A, Metabolite B (4-OHT), Metabolite D (catechol/dihydroxytamoxifen), Metabolite E, Metabolite F, Metabolite Y, N-desmethyl-tamoxifen and tamoxifen N-oxide. This paper also reports that N-desmethyltamoxifen (and not 4-OHT) is the major metabolite of tamoxifen in human patients. *Id.* at 126. Many of these metabolites have significant anti-estrogen activities and inhibit estrogen signal transduction. Again, the multiplicity of biologic activities and active metabolites makes the prediction that 4-OHT would be either the optimal choice or even an obvious choice uncertain.

15. Different tamoxifen metabolites have different activities. For example, Jordan *et al.*, *supra*, report that 4-OHT has a strong binding affinity for the estrogen receptor and exhibits anti-estrogen activity *in vivo* in humans. *Id.* at 130. In contrast, dihydroxytamoxifen has an estrogen receptor binding affinity that is similar to estradiol, and is a "partial agonist with anti-estrogenic properties" in a murine uterine weight test, "although both tamoxifen and dihydroxytamoxifen are full estrogen agonists in the mouse." *Id.* at 131. On the other hand, Metabolite E appears to be a "weak estrogen" in the immature rat uterine weight test, while Metabolite Y "is a weak antiestrogen with partial estrogenic activity in the rat uterus." *Id.* As recognized by the authors at the time of its publication in 1982, "our knowledge is still incomplete with regard to the identification and pharmacological role of individual metaholites in target tissues and tumors." Thus, for example, even knowing that a metabolite (such as 4-OHT) has a strong binding affinity for the estrogen receptor may not be predictive of pharmacological activity *in vivo*, let alone be predictive of specific activity in a clinical condition such as mastalgia or breast density.

16. One metabolite that has received a significant amount of attention for its pharmacological potential is 4-hydroxy-N-desmethyltamoxifen, also known as endoxifen. By 1982, endoxifen had been discovered and reported to have an estrogen receptor binding affinity many times greater than tamoxifen. *See, e.g.*, Robertson *et al.*, *supra*, at page 8, Table 1. Data reported by Robertson show endoxifen to have a

relative binding affinity for the rat uterine estrogen receptor of 143, as compared to a relative binding affinity of 2 for *trans (Z)* tamoxifen. (For comparison, 4-OHT is reported to have a relative binding affinity of 285, while N-desmethyltamoxifen is reported to have a relative binding affinity of 3.) By 1988, Lien *et al.*, *Cancer Res.* 48: 2304-08 (1988) (copy attached), had hypothesized that endoxifen is a "secondary" metabolite of tamoxifen, formed from either 4-OHT or N-desmethyltamoxifen. In view of Jordan's report that N-desmethyltamoxifen (not 4-OHT) is the major metabolite of tamoxifen in human patients (discussed above), those working in the field had reason to expect that endoxifen is produced via a metabolic pathway from N-desmethyltamoxifen. Indeed, this is the current state of thinking, as reflected in the diagram from Desta, *supra*, set forth above. This diagram shows that, as currently understood, the major metabolic pathway from tamoxifen to endoxifen goes through N-desmethyltamoxifen, with only a minor pathway through 4-OHT.



17. By 1990, Lien *et al.*, *Cancer Res.* 50: 5851-87 (1990) (copy attached), had reported serum concentration studies showing that serum concentrations of endoxifen are generally higher than 4-OHT. For example, Table 3 at page 5854 reports mean serum concentrations of endoxifen (Metabolite BX) that are more than 4 times greater than

that of 4-OHT (Metabolite B). By 1992, endoxifen was being studied as having "biological importance, with an affinity for the estrogen receptor several-fold higher than that of *trans*-tamoxifen." Lonning, *supra*, at 335. These data suggest that endoxifen might have been considered to be the "optimal" alternative to tamoxifen.

18. Accordingly, by the 2002 priority date of the applications, 4-OHT was not the only active metabolite of tamoxifen with promise for pharmacological activity and was not necessarily the most promising or viable choice for further study.

19. While the Examiners have focused on the anti-estrogen activity of 4-OHT, it is important to understand that anti-estrogen activity does not always correlate with pharmacological efficacy, and that an anti-estrogen agent that is effective for treating one condition may not be effective for treating another condition. This point is made quite plainly in Gradishar & Jordan, *J. Clin. Oncol.* 15: 840-52 (1997) (copy attached), which emphasizes at page 841 that "[r]eceptor binding and biologic activity are now viewed as two separate functions." Indeed, the authors note that one challenge facing the field is the development of an antiestrogen that will have antiestrogen activity in the breast, pro-estrogen activity in the central nervous system, lower cholesterol, and maintain bone density (pro-estrogenic) without causing uterine stimulation. Gradishar & Jordan, *supra*, Fig. 2.

20. Gradishar & Jordan, *supra*, summarizes the then-current state of the art with respect to the clinical potential of new antiestrogens, including the tamoxifen metabolite 3-hydroxytamoxifen, also known as droloxifene, and other tamoxifen derivatives. The story of droloxifene taught those working in the field that estrogen receptor binding activity is not necessarily predictive of biological activity. By 1994, this tamoxifen metabolite was proving to he a promising candidate for breast cancer therapy. Droloxifene binds the estrogen receptor with 10-60 times the affinity of tamoxifen, and was found to be safer than tamoxifen in animal studies. *See, e.g.*, Hasmann *et al., Cancer Lett.* 84: 101-16 (1994) (copy attached). Gradishar & Jordan, *supra*, at page 841, characterized droloxifene as reflecting "[t]he principle of an antiestrogen with high affinity for the [estrogen receptor]." However, by 1998, it was

reported that "interim data from a phase III trial "was discouraging, showing that droloxifene 'offered no benefit beyond [tamoxifen].'" McNeil, *J. Nat'l Cancer Inst.* 90: 956-57 (1998) (copy attached), at 957. In 2002, the investigators published the results of that trial, which was halted early, concluding that "[d]roloxifene was significantly less effective than tamoxifen overall." Buzdar *et al.*, *Breast Cancer Res. & Treat.* 73: 161-75 (2002), Abstract. Indeed, the investigators stated that "no further development of droloxifene will be undertaken." *Id.* at 174. This example documents that identifying a high affinity anti-estrogenic metabolite of tamoxifen does not predict in any way utility, let alone a specific "anti-estrogenic" clinically beneficial effect in a specific patient population.

21. Raloxifene provides another example of the high level of unpredictability in this field. Raloxifene is a selective estrogen receptor modulator with anti-estrogen activity. It has a similar activity as tamoxifen in treating breast cancer in menopausal women, but is reported to have mixed effects on breast density. *See, e.g.*, Chlebowski & McTiernan, *J. Nat'l Cancer Inst.* 95: 4-5 (2003) ("Tamoxifen has fairly consistently decreased breast density, whereas the effects of raloxifene on breast density have been mixed.") (copy attached). For example, Freedman *et al.*, *J. Nat'l Cancer Inst.* 93: 51-56 (2001) (copy attached), presents data indicating that raloxifene "did not increase breast density," but achieved only a 1.5% or 1.7% (depending on the dose) decrease in breast density, where the placebo achieved a 1.3% decrease. Christodoulakos *et al.*, *Menopause* 9: 110-16 (2002) (abstract attached), reports on a study where 6.3% of patients treated with raloxifene showed an increase in breast density, where no increase was seen in the control group.

22. So, while the Examiners' "obviousness" positions are based on the known estrogen receptor binding activity of 4-OHT, those working in the field knew by 1998 (well before the patent applications were filed) that estrogen receptor binding activity was not necessarily predictive of clinical efficacy.

23. It is also important to keep in mind that tamoxifen and other SERMs and anti-estrogen agents can have different activities in pre-menopausal women than in post-

menopausal women, who lack the ability to produce endogenous estrogen. For example, Gradishar & Jordan, *supra* at 845, reported that droloxifene achieved most of its positive responses in "perimenopausal or post-menopausal patients." Buzdar *et al.*, *supra* at Abstract, confirmed this understanding, reporting that both tamoxifen and droloxifene were "less effective in pre-menopausal women with receptor-positive disease compared to post-menopausal women." Thus, results obtained in post-menopausal women are not predictive of results that will be obtained in pre-menopausal women, who might increase production of endogenous estrogen in response to tamoxifen's anti-estrogen activity. This is particularly important for the treatment of mastalgia, which only occurs in pre-menopausal patients.

24. In summary, by 2002, those working in the field knew that selective estrogen receptor modulators were highly unpredictable, can have different (even opposite) activities in different tissues, and can have different effects in pre- vs. post menopausal women. Thus, it was not possible to predict from studies with tamoxifen, or from studies of 4-OHT in different patient populations, that 4-OHT could be used to treat mastalgia or reduce breast density.

25. I provide the following comments in response to the Office Action mailed August 23, 2007, in U.S. Patent Application 10/734,640, in particular to the citation of Pujol *et al.*, *Cancer Chemother. Pharmacol.* 36: 493-98 (1995) and Mauvais-Jarvis, *Curr. Ther. Endocrin. Metab.* 280-84 (1988) and Mauvais-Jarvis, Senologie & Pathologie Mammaire, 4eme Cong. Int'l, 128-32 (1986).

26. The Office Action, at page 3, states that "Pujol et al. teaches a percutaneous administration of . . . [4-OHT] in a hydroalcoholic get to breast areas for the treatment of breast cancer," but this characterization of this Pujol reference is misleading. Pujol reports a Phase I study that measured the concentration of 4-OHT in the breast tissue of breast cancer patients after percutaneous administration of a 4-OHT hydroalcoholic gel. The authors found a lower breast tissue concentration as compared to oral tamoxifen and concluded that "at the doses described in this study, percutaneous 4-OH-TAM ***cannot be proposed as an alternative tamoxifen treatment.***" Pujol,

Abstract (emphasis added). Thus, contrary to the interpretation suggested by the Office Action, this Pujol reference does not suggest that percutaneous 4-OHT can be successfully used in place of oral tamoxifen to treat breast cancer, let alone to treat mastalgia.

27. The Office Action, at page 3, cites page 281 of Mauvais-Jarvis, *Curr. Ther. Endocrin. Metab.* 280-84 (1988), for teaching that "tamoxifen has been proposed for the treatment of benign breast disease [including mastalgia]," and that percutaneous 4-OHT might "avoid the systemic effects of the oral administration of tamoxifen." The statements in Mauvais-Jarvis regarding the potential efficacy of percutaneous 4-OHT are entirely speculative, however. The only data cited pertain to studies showing that when 4-OHT is topically applied to the breast it is "absorbed through the skin and is predominantly concentrated into subcellular fractions of breast tissue. No efficacy experiments support the hypothesis that percutaneous 4-OHT could be useful against benign breast disease. Indeed, the cited page of Mauvais-Jarvis states that "[t]his possible therapeutic approach is under investigation and is not yet available." Thus, contrary to the position set forth in the Office Action, this Mauvais-Jarvis reference does not provide the person skilled in the field with a sufficient scientific basis to reasonably predict that 4-OHT could be used to treat mastalgia.

28. The Office Action, at pages 5 & 6, cites pages 129 and 130 of Mauvais-Jarvis, SENOLOGIE & PATHOLOGIE MAMMAIRE, 4eme Cong. Int'l, 128-32 (1986), for teaching that "tamoxifen has been proposed for the treatment of benign breast disease [including mastalgia]," and that percutaneous 4-OHT might "avoid the systemic effects of the oral administration of tamoxifen." The statements in this Mauvais-Jarvis paper are almost identical to the statements in Mauvais-Jarvis, *Curr. Ther. Endocrin. Metab.* 280-84 (1988), and are likewise entirely speculative, and based only on phase I data showing that topically applied 4-OHT localizes in subcellular breast tissue. Thus, contrary to the position set forth in the Office Action, this Mauvais-Jarvis reference does not provide the person skilled in the field with a sufficient scientific basis to reasonably predict that 4-OHT can be used to treat mastalgia.

29. I provide the following comments in response to the Office Action mailed August 4, 2007, in U.S. Patent Application 10/734,644, in particular to the citation of Mauvais-Jarvis *et al.*, U.S. Patent 4,919,937 and Mauvais-Jarvis *et al.*, *Cancer Res.* 46: 1521-25 (1986).

30. The Office Action, at page 5, cites the Mauvais-Jarvis patent for teaching "a method of treating conditions of the breast comprising administering percutaneously an aqueous gel comprising [4-OHT]," where the conditions of the breast include "benign and cancerous conditions of the breast." The Mauvais-Jarvis patent, at column 4, states that "the drug described [4-OHT] finds application in the treatment of conditions of the breast, especially benign and even cancerous conditions of the breast." To the extent that the Examiner understands this general statement to provide a reasonable expectation of success in being able to use 4-OHT to treat the specific condition of breast density, e.g., to reduce breast density, I must respectfully disagree. There is simply no data presented in the Mauvais-Jarvis patent indicating that 4-OHT would be effective to reduce breast density.

31. The Office Action, at page 5, cites Mauvais-Jarvis *et al.*, *Cancer Res.* 46: 1521-25 (1986), for showing that the "percutaneous administration of 4-OHT results in an equal yield of the cis and trans isomers . . . from breast tissue." This Mauvais-Jarvis reference reports on the tissue concentration and metabolism of *Z* 4-OHT after local administration to the breasts of female breast cancer patients, and does not present any data indicating that 4-OHT would be effective to reduce breast density.

32. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that willful, false statements may jeopardize the validity of the application or any patent issued thereon.

2-19-08
DATE

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Comprehensive Evaluation of Tamoxifen Sequential Biotransformation by the Human Cytochrorne P450 System in Vitro: Prominent Roles for CYP3A and CYP2D6

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ABSTRACT

We performed comprehensive kinetic, inhibition, and correlation analyses in human liver microsomes and experiments in expressed human cytochromes P450 (P450s) to identify primary and secondary metabolic routes of tamoxifen (TAM) and the P450s catalyzing these reactions at therapeutically relevant concentrations. *N*-Desmethyl-TAM formation catalyzed by CYP3A4/5 was quantitatively the major primary metabolite of TAM; 4-hydroxy-TAM formation catalyzed by CYP2D6 (and other P450s) represents a minor route. Other minor primary metabolites include α -, 3-, and 4'-hydroxyTAM and one unidentified metabolite (M-I) and were primarily catalyzed by CYP3A4, CYP3A5, CYP2B6/2C19, and CYP3A4, respectively. TAM secondary metabolism was examined using *N*-desmethyl- and 4-hydroxy-TAM as intermediate substrates. *N*-Desmethyl-TAM was predominantly biotransformed to α-hydroxy *N*-desmethyl-, *N*-didesmethyl-, and 4-hydroxy *N*-desmethyl-TAM (endoxifen), whereas 4-hydroxy-TAM was converted to 3,4-dihydroxyTAM and endoxifen. Except for the biotransformation of *N*-desmethyl-TAM to endoxifen, which was exclusively catalyzed by CYP2D6, all other routes of *N*-desmethyl- and 4-hydroxy-TAM biotransformation were catalyzed predominantly by the CYP3A subfamily. TAM and its primary metabolites undergo extensive oxidation, principally by CYP3A and CYP2D6 to metabolites that exhibit a range of pharmacological effects. Variable activity of these P450s, brought about by genetic polymorphisms and drug interactions, may alter the balance of TAM effects in vivo.

The selective estrogen receptor modulator tamoxifen (TAM) was first approved in 1977 by the U.S. Food and Drug Administration for the treatment of women with metastatic breast cancer and in ensuing years for adjuvant treatment of breast cancer (Osborne, 1998). Currently, TAM is an established hormonal treatment for all stages of estrogen receptor (ER)-positive breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1998) and is widely used as a chemopreventive agent in women at risk for developing the disease (Fisher et al., 1998). Ancillary benefits of TAM may include amelioration of cardiovascular and bone disease in women (Osborne, 1998). However, there is wide interindividual variability in the clinical efficacy and side effects of TAM: some patients may be refractory to TAM, and many develop resistance with ongoing treatment (Osborne, 1998; Clarke et al., 2001) and a significant proportion of patients experience side effects that include hot flashes, thromboembolic events, and gynecologic complications (Osborne, 1998). The mechanisms underlying variable response to TAM have been the subject of intense study but remain obscure. Since there is compelling evidence that TAM is converted to antiestrogenic metabolites that are more potent than TAM itself, one hypothesis is that altered patterns of metabolism of TAM and/or its primary metabolites might contribute to interindividual variability.

Theories about TAM metabolism and response have been linked since Jordan et al. demonstrated that high first-pass metabolism of TAM results in a significant increase in its activity and characterized the first active *primary* metabolite, 4-hydroxy-TAM (Jordan, et al. 1977; Jordan, 1982). This metabolite has been shown to possess a high affinity for ERs and 30- to 100-fold more potency than TAM in suppressing estrogen-dependent cell proliferation (Borgna and Rochefort, 1981; Robertson et al., 1982; Coezy et al., 1982; Jordan, 1982). Thus, TAM has been referred to as a pro-drug that requires activation to exert its effects. Recently, we have

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Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.104.065607.

ABBREVIATIONS: TAM, tamoxifen; ER, estrogen receptor; endoxifen, 4-hydroxy-*N*-desmethyl-tamoxifen; P450, cytochrome P450; HPLC, high-performance liquid chromatography; HLM, human liver microsome; CL_{int}, intrinsic clearances; TEPA, *N,N',N''*-triethylene phosphoramide.

demonstrated that 4-hydroxy-*N*-desmethyl-TAM (endoxifen), a *secondary* metabolite of TAM, exhibits potency similar to 4-hydroxy-TAM with respect to ER binding affinity, suppression of estrogen-dependent cell growth, and gene expression (Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2004).

In vitro and in vivo human studies have demonstrated that TAM undergoes extensive oxidation predominantly by the cytochrome P450 (P450) system to several primary and secondary metabolites (Lonning et al., 1992; Poon et al., 1993; White, 2003). Clinical studies suggest that the plasma concentrations of TAM and its metabolites vary widely among patients (Lonning et al., 1992; Stearns et al., 2003). It is likely that variable activity of those P450s involved alter the pattern of metabolism of TAM, leading to differences in systemic exposure of TAM and/or one or more of its active metabolites.

Several studies have attempted to define the primary metabolism of TAM in humans. Demethylation of the aminoethoxy side chain to *N*-desmethyl-TAM appears to be the main route of TAM metabolism (Lonning et al., 1992; Stearns et al., 2003). 4-Hydroxy-TAM is a relatively minor metabolite, but it has been studied by a number of investigators because it is a more potent antiestrogen than TAM (Jordan et al., 1977; Borgna and Rochefort, 1981; Coezy et al., 1982; Robertson et al., 1982). Other human primary metabolites that include α-hydroxy-TAM and TAM *N*-oxide have been identified (Lonning et al., 1992; White, 2003). Published reports implicate multiple P450s in TAM *N*-demethylation, 4-hydroxylation, and α-hydroxylation (e.g., Jacolot et al., 1991; Crewe et al., 1997, 2002; Dehal and Kupfer, 1997). Despite the conduct of extensive in vitro studies to characterize TAM primary metabolism, critical evaluation of the published literature highlights the lack of a thorough quantitative understanding that will allow better prediction of TAM clearance or production of its active antiestrogens or toxic metabolites in vivo.

There is evidence that the primary metabolites of TAM are further oxidized by the human P450 system to a variety of important metabolites (Lonning et al., 1992; Dehal and Kupfer, 1999; Stearns et al., 2003; White, 2003; Coller et al., 2004). The potential significance of TAM secondary metabolism was suggested by recent studies that identified endoxifen as a potent antiestrogen metabolite of TAM (Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2004). Since the plasma concentration of this metabolite in breast cancer patients has been reported to be much higher (over 6-fold) than that of 4-hydroxy-TAM (Lien et al., 1990; Stearns et al., 2003), it may also play an important role in vivo. Despite this, little information is available regarding TAM sequential metabolism to its secondary metabolites. Recently, we reported an association between CYP2D6 genotype and endoxifen formation in breast cancer patients (Stearns et al., 2003), but the contribution of this and/or other enzymes to its production has not been subject to careful study. Several secondary metabolites other than endoxifen have been identified in humans. Knowledge of sequential metabolic pathways leading to these metabolites and identification of the specific P450s involved remains essential to a full understanding of the human metabolism of this important drug.

In the present study, we carried out a comprehensive series of kinetic, correlation, and inhibition studies in human liver microsomal preparation and expressed P450s to characterize the metabolism of TAM to both primary and secondary metabolites.

Materials and Methods

Chemicals. Endoxifen was synthesized from [Z]-4-hydroxy-TAM (98% pure from Sigma-Aldrich, St. Louis, MO) as described in our recent publications (Stearns et al., 2003; Johnson et al., 2004). Z-TAM, Z-4-hydroxy-TAM, propranolol, troleandomycin, ketoconazole, diethyldithiocarbamate, quinidine sulfate, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and β-$NADP^+$ were purchased from Sigma-Aldrich. For initial metabolite identification, *N*-desmethyl-TAM, *N*-didesmethyl-TAM, and TAM *N*-oxide that were kindly provided by Dr. Irving W. Wainer (Laboratory of Clinical Investigation, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD) were used. For actual incubation experiments, *N*-desmethyl-TAM HCl and α-hydroxy-TAM were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada), or *N*-desmethyl-TAM was synthesized from [Z]-tamoxifen (98% pure from Sigma-Aldrich) using the same protocol as described elsewhere (Stearns et al., 2003). ThioTEPA was purchased from the U.S. Pharmacopoeia Convention (Rockville, MD). Sulfaphenazole and furafylline were purchased from Ultrafine Chemicals (Manchester, UK). Metabolite E [*trans*-1(4-hydroxyphenyl)1,2-diphenylbut-1-ene] was a generous gift of Dr. Michael D. Johnson (Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC). 4'-Hydroxy-TAM was a generous gift from Dr. Martin Lennard (Section of Molecular Pharmacology and Pharmacogenetics, University of Sheffield, Sheffield, UK). 3-Hydroxy-TAM was kindly provided by Dr. Stephen D. Hall and Dr. David R. Jones (Division of Clinical Pharmacology, Indiana University School of Medicine, Indianapolis, IN). Omeprazole was a generous gift from Dr. Tommy Andersson (Clinical Pharmacology, Astra Hässle AB, Mölndal, Sweden). All other reagents were of HPLC grade.

Human Liver Microsomes (HLMs) and Expressed P450s. Most of the HLMs used in this study and *Baculovirus* insect cell expressed human P450 isoforms (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) (with reductase) were purchased from BD Biosciences (Franklin Lakes, NJ). Some HLMs were prepared from human liver tissues that were medically unsuitable for liver transplantation by differential centrifugation using standard procedures. Microsomal pellets were resuspended in a reaction buffer to a protein concentration of 10 mg/ml (stock). Protein concentrations were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard. All microsomal samples were kept at −80°C until use.

Incubation Conditions. Pilot experiments were performed in HLMs using TAM, *N*-desmethyl-TAM, and 4-hydroxy-TAM as substrates to identify TAM primary and secondary metabolites and to optimize conditions for incubation and HPLC analysis. Each substrate (1 mg/ml) was prepared in methanol and serially diluted with methanol to the required concentration. Prior to metabolic incubation, the methanol was evaporated to dryness under reduced pressure using a Speedvac SC110 Model RH40-12 (Savant Instruments, Holbrook, NY). Duplicate mixtures of 10 μM of each substrate reconstituted in a phosphate reaction buffer (pH, 7.4) and a NADPH-generating system (1.3 mM β-$NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/ml glucose 6-phosphate dehydrogenase) were preincubated at 37°C for 5 min before the reaction was initiated by the addition of HLMs, 0.5 mg protein/ml (final volume, 250 μl; all values are final concentrations). After a 60-min incubation period, the reaction was terminated by addition of cold acetonitrile (100 μl) and vortex mixing. Samples were centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkmann Instruments, Westbury, NY) after addition of an internal standard (20 μl of 100 μg/ml propranolol). An aliquot of supernatant was injected into the HPLC system. Negative control incubations were run in parallel

and included no incubation, no substrate, no cofactor, or no HLMs (bovine serum albumin was used instead). All experiments were performed under dim light and in brown Eppendorf tubes to minimize photodegradation of TAM and its metabolites.

HPLC Analysis. Metabolites in microsomal incubates of TAM and *N*-desmethyl- and 4-hydroxy-TAM were measured using an HPLC method developed previously by our group (Lee et al., 2003, Stearns et al., 2003) with slight modification. Briefly, the incubation sample was injected onto a semipermeable surface cyano guard loading column (1.0- × 0.46-cm i.d.; 5 μm, 100Å) and washed with 1 ml/min deionized water followed by a mobile phase composed of 35% acetonitrile in 20 mM potassium phosphate buffer (pH, 3) at a flow rate of 1 ml/min. The duration of washing off the guard column with water and the mobile phase was reduced from 4 min in the previous publication (Lee et al., 2003) to below 2 min in the present study. This modification significantly improved the sensitivity of the assay by increasing the yield of metabolites produced in small quantities in HLMs. Using the mobile phase, the eluant was directed (switched) through a Rexchrom cyano analytical C_{18} column (250- × 4.6-cm i.d.; 5 μm, 100Å; Regis Chemical, Morton Grove, IL) guarded with a Rexchrom 10- × 3-mm C18 guard column (Regis Chemical) to an ICT Beam Boost postcolumn photoreactor supplied with a 5-m reaction coil and a 254 UV lamp (Advanced Separation Technologies, Whippany, NJ) where the photoreaction converts tamoxifen and its metabolites to highly fluorescent phenanthrene derivatives. HPLC instruments were controlled by CLASS-VP version 7.1.1 SP1 Chromatographic Software (Shimadzu Scientific Instruments Inc., Columbia, MD) and included a Shimadzu Solvent Delivery Module SCL-10A VP, Autoinjector SIL-10AD VP, Spectrofluorometric detector RF-10A XL (set at an excitation wavelength of 256 nm and emission wavelength of 380 nm) and System Controller SCL-10A VP (Shimadzu, Kyoto, Japan).

Identification of Metabolites. Using the incubation and HPLC conditions described above, we observed several well separated fluorescent HPLC peaks in the incubation mixture consisting of the substrate (TAM, *N*-desmethyl- or 4-hydroxy-TAM), a NADPH-generating system, and HLMs, but not in the negative control experiments. The identity of each metabolite peak was confirmed as follows. First, the retention times of the peaks formed in microsomal incubates were compared with peaks of authentic TAM primary or secondary metabolite standards when available. The retention times of the reference metabolites were tested after direct injection or after adding them to incubation mixture that did not contain active microsomes. Second, selected ion recording scans of the microsomal incubates of TAM were performed with the help of liquid chromatography-mass spectrometry. Metabolites were separated using a Rexchrom cyano analytical C_{18} column (250- × 4.6-cm i.d.; 5 μm, 100Å; Regis Chemical) guarded with a Rexchrom 10- × 3-mm C18 guard column (Regis Chemical) and a mobile phase that consisted of 50 mM ammonium acetate/methanol/acetonitrile (50:25:25, *v/v/v*). The flow rate was 0.2 ml/min. The mass spectrometer (PE/SciFx API100) was operated in positive ion mode and tuned for optimal resolution over the mass range by PE/SciEx proprietary LC2Tune program software. The exact conditions were: capillary voltage, 5300 V; orifice voltage, 30 V; and mass range of 150 to 450. The mass spectrometric data obtained in microsomal incubates were compared with the molecular masses of reference metabolites run in parallel or with the molecular masses of metabolite profiles of tamoxifen previously reported in humans (Lonning et al., 1992; Poon et al., 1993; White, 2003). Additional information on whether a metabolite peak represents primary or secondary metabolite was obtained by comparing the peaks of TAM microsomal incubates with those obtained from incubation of its primary metabolites: *N*-desmethyl-, 4-hydroxy-, or α-hydroxy-TAM.

Linear Conditions for Incubation. The duration of incubation and the protein concentrations used above were useful for initial experiments aimed at identification of TAM primary and secondary metabolites, but they may not represent linear conditions. Several investigators have used long duration of incubation, probably offset low assay sensitivity for metabolites that are normally formed at a slower rate. Given that TAM undergoes sequential metabolism, longer duration of incubation may increase the likelihood of secondary metabolism, which then may preclude precise estimation of kinetic parameters and of contribution of specific metabolic routes to the overall clearance or activation of the drug. The need for optimizing microsomal protein concentrations during TAM metabolism studies has been emphasized elsewhere (Crewe et al., 1997). To determine conditions that are linear for time of incubation and protein concentration, 10 μM each substrate (TAM, *N*-desmethyl-TAM or 4-hydroxy-TAM) was incubated in HLMs (0–2 mg protein /ml) and a NADPH-generating system at 37°C across a range of incubation times (0–90 min). Further processing of the samples was the same as the procedures described above (see *Incubation Conditions* and *HPLC Analysis*). Based on the results obtained, a 10-min incubation and a final microsomal protein concentration of 0.1 mg/ml represented conditions that are linear and minimize secondary metabolism of the substrates and were used in the subsequent experiments.

The concentration of each metabolite was measured by comparing the metabolite peak to a standard curve obtained using known concentrations of the respective authentic metabolite standard. Authentic metabolite standards were not available to us for few metabolites identified (M-1, 3,4-dihydroxy-TAM, and α-hydroxy *N*-desmethyl-TAM), and these metabolites were quantified using standard curves obtained from a metabolite standard that had close structural similarity and HPLC properties with the metabolite in question. The limitation of this approach is that the fluorescent intensity between the metabolite and the compound used to quantify it may be different as a result of altered chromophore. As a result, although the K_m values for the formation of these metabolites could be estimated appropriately, actual formation rates of the metabolites could not be estimated precisely. Thus, the formation rates presented for these metabolites as picomoles per minute per milligram of protein (or picomoles per minute per picomoles of P450) should be viewed more appropriately as apparent velocities (arbitrary unit per minute per milligram of protein or picomoles of P450) where an arbitrary unit = 1000 × (metabolite AUC/internal standard AUC)/slope of the standard curve.

Kinetic Analyses in HLMs. Full kinetic analyses for the metabolism of TAM and its primary metabolites, *N*-desmethyl- and 4-hydroxy-TAM, were determined in characterized HLMs (n = 3–4). A range of substrate concentrations (0–100 μM) was incubated for 10 min at 37°C with a protein concentration of 0.1 mg protein/ml and a NADPH-generating system. Further processing of the samples and HPLC assay were the same as described above.

Correlation Analyses in HLMs. To determine the correlation between the metabolism of TAM, *N*-desmethyl- or 4-hydroxy-TAM, and the activity of individual P450 isoform in a panel of characterized HLMs (n = 10–11), the respective substrate (10 μM) was incubated at 37°C for 10 min with HLMs (0.1 mg protein/ml) and a NADPH-generating system. Information on the activity of each P450 isoform, measured by specific reaction marker, and on P450 contents was provided by the supplier of the HLMs studied (see http://www.gentest.com) and has been detailed in our previous publication (Ward et al., 2003).

Inhibition Experiments. Formation rates of metabolites from TAM and *N*-desmethyl- and 4-hydroxy-TAM (10 μM) were evaluated in the absence (control) and presence of known P450 isoform-specific inhibitors. The inhibitors used were 10 μM furafylline (CYP1A2), 50 μM thioTEPA (CYP2B6), 20 μM sulfaphenazole (CYP2C9), 5 μM omeprazole (CYP2C19), 1 μM quinidine (CYP2D6), 50 μM diethyldithiocarbamate (CYP2E1), and 1 μM ketoconazole and 50 μM troleandomycin (CYP3A4/5). For competitive inhibitors, each substrate was prewarmed for 5 min at 37°C with or without the inhibitor and with a NADPH-generating system. HLMs (0.1 mg protein/ml) were added to initiate the reaction and incubated at 37°C for 10 min. For mechanism-based inhibitors (troleandomycin, furafylline, and thio-

TEPA), the inhibitors and controls were first preincubated in the presence of a NADPH-generating system and HLMs at 37°C for 15 min before the reaction was initiated by addition of the respective substrate. The inhibitors were studied at concentrations chosen to be selective for the respective P450 isoforms (Ward et al., 2003). Inhibited formation rates of metabolites were compared with those of control (with no inhibitor present), and results are expressed as a percentage of control activity.

Expressed Human P450s. To further probe the specific isoforms involved in tamoxifen primary and secondary metabolism, 10 μM of each substrate (TAM or *N*-desmethyl- or 4-hydroxy-TAM) was incubated with microsomes from expressed human CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and CYP3A5 (52–104 pmol P450/ml in phosphate reaction buffer, pH 7.4) at 37°C for 10 min (final incubation volume = 250 μl). All other conditions were the same as those described for HLMs. For selected P450 isoforms, kinetic parameters were estimated by incubating 0 to 100 μM of the substrates with expressed P450s (52–104 pmol of P450/ml) and a NADPH-generating system for 10 min at 37°C.

Data Analysis. Apparent kinetic constants were estimated by nonlinear regression analysis using WinNonlin Software Version 3.1 (Pharsight, Mountain View, CA). Formation rates (V) of metabolites versus substrate concentrations (C) were fit to a single-site ($V = V_{max} \cdot C/(K_m + C)$), two-site ($V = [V_{max1} \cdot C/(K_{m1} + C)] + [V_{max2} \cdot C/(K_{m2} + C)]$), or substrate inhibition ($V = V_{max}/(1 + K_m/C + C/K_{si})$) equation. The model that fit best the data was selected based on visual inspection of the Eadie-Hofstee plots, the dispersion of residuals, and standard errors of the parameter estimates. In vitro intrinsic clearances (CL_{int}) were given as V_{max}/K_m. Pearson's correlation or linear regression tests were performed using GraphPad Prism Software version 3.1 (GraphPad Software Inc., San Diego, CA) to calculate correlation coefficients. Formations of metabolites in HLMs with high and low activity of P450s were compared using unpaired two-tailed Student's t test. $P < 0.05$ was considered statistically significant. Data are presented as mean ± S.D. (n = 3–4 HLMs) or as averages of duplicate experiments.

Results

TAM Oxidation to Its Primary Metabolites in HLMs and Expressed P450s

A series of experiments were performed in HLMs and expressed P450s to characterize TAM oxidation to its primary metabolites in conditions that minimize secondary metabolism.

Identification of TAM Primary Metabolites in HLMs. HPLC traces of tamoxifen and its metabolites from in vitro incubation of TAM with HLMs showed several metabolite peaks that depended on a NADPH, duration of incubation, and microsomal protein and substrate concentrations (data not shown). The metabolite peaks at the retention times of 47, 31, 30, 29, and 18.3 min, respectively, were identified as *N*-desmethyl-, 4′-hydroxy-, 3-hydroxy-, 4-hydroxy-, and α-hydroxy-TAM. 4′-Hydroxy-TAM has been previously detected in the rat (Ruenitz et al., 1984), and a recent study reported that expressed human P450s catalyze its formation (Crewe et al., 2002), but this is the first evidence that this metabolite is formed in HLMs. To our knowledge, 3-hydroxy-TAM was not reported as primary metabolite of TAM in humans. Two other metabolite peaks were noted at retention times of 15.5 and 16.8 min. The metabolite peak at 15.5 min had properties consistent with a secondary metabolite, and subsequent experiments (see below) showed that it concurs with α-hydroxy *N*-desmethyl-TAM. The metabolite at 16.8 min (designated as M-I) has properties that are consistent with a primary metabolite, but its identity remains unknown. Other primary metabolites that appeared at retention times > 60 min such as TAM *N*-oxide and metabolite E [*trans*-1(4-hydroxyphenyl)1,2-diphenylbut-1-ene] or those that were formed at very slow rates were not further characterized. Secondary metabolites that include *N*-didesmethyl-TAM (retention time, 45 min) and endoxifen (retention time, 25 min) were also noted when TAM was incubated for 60 min. However, the secondary metabolites of TAM were not observed at 10 min of incubation.

Kinetic Analyses of TAM Primary Metabolism in HLMs. Several in vitro studies have suggested involvement of multiple P450 isoforms in TAM primary metabolism, but extrapolations to clinical conditions have been limited in part by the lack of appropriate kinetic analyses to help identify (and estimate the precise contributions of) the P450s that are responsible at therapeutically relevant concentrations. We have addressed this issue by conducting comprehensive kinetic analyses of TAM primary metabolism in three HLMs characterized for their P450 activity in incubation conditions that minimize subsequent metabolism of the primary metabolites formed. The kinetic parameters for the metabolism of TAM to *N*-desmethyl- and 4-hydroxy-TAM illustrated in Table 1 were estimated by fitting formation rates versus TAM concentrations to a two-site binding equation with use of a nonlinear regression analysis (see *Data Analysis*). Accordingly, the Eadie-Hofstee plots of these data (formation rates versus rates/substrate concentration) showed biphasic kinetics (data not shown). Our data suggest the participation of at least two enzymatic activities: high affinity (low capacity) (K_{m1} and V_{max1}) and low affinity (high capacity) (K_{m2} and V_{max2}). The high-affinity (low-capacity) system with average K_m values <3 μM and a >10-fold higher V_{max1}/K_{m1} than that of V_{max2}/K_{m2} (Table 1) is probably relevant at therapeutic

TABLE 1

Kinetic parameters for the metabolism of TAM to *N*-desmethyl-TAM and 4-hydroxy-TAM in HLMs (n = 3)

The HLMs used were characterized with respect to the activity of individual P450: CYP2D6 activity (measured by bufuralol 1′-hydroxylation) in HG23 and HG112 was 135 and 24.5 pmol/min/mg of protein, respectively, whereas in HG06 it was undetectable. The activity of CYP3A (measured by testosterone 6β-hydroxylation) in HG112, HG23, and HG06 was 17,205, 3320, and 3000 pmol/min/mg of protein, respectively. Formation rates of metabolites versus TAM concentrations were best fit to a two-site binding equation using a nonlinear regression model (see *Data Analyses*).

HLMs	TAM to *N*-Desmethyl-TAM						TAM to 4-Hydroxy-TAM					
	V_{max1}	K_{m1}	V_{max1}/K_{m1}	V_{max2}	K_{m2}	V_{max2}/K_{m2}	V_{max1}	K_{m1}	V_{max1}/K_{m1}	V_{max2}	K_{m2}	V_{max2}/K_{m2}
HG06	33.3	1.1	31.1	1740	611	2.9	0.7	1.3	0.6	0.8	16.2	0.05
HG23	13.5	2.1	6.4	150	55.6	2.7	3.8	0.7	5.5	4.3	46.0	0.09
HL112	142	3.3	43.0	1719	910	1.9	1.2	0.8	1.5	4.2	11.5	0.4
Mean	62.9	2.2	26.8	1203	526	2.5	1.92	0.9	2.0	3.1	24.6	0.17
±S.D.	69.2	1.1	18.7	912	434	0.5	1.67	0.3	2.6	2.0	18.4	0.17

V_{max}, pmol/min/mg of protein; K_m, μM; and V_{max}/K_m, μl/min/mg of protein.

doses of the drug. The mean steady-state plasma concentrations of TAM following therapeutic doses (20–40 mg/day orally) (Lonning et al., 1992; Stearns et al., 2003) or even at high doses (120 mg/m^2 to treat brain tumors) (Ducharme et al., 1997) do not exceed 2 and 8 μM, respectively. There is evidence that the concentrations of TAM and its metabolites are notably higher in human liver tissue relative to plasma, in some cases up to 60-fold (Lien et al., 1991). Even then, given the high (over 98%) plasma protein (albumin) binding of TAM (Lien et al., 1989), the unbound TAM concentration is unlikely to exceed 10 μM.

N-Desmethyl- and 4-hydroxy-TAM have been described in the literature as major and minor metabolites of TAM, respectively (Lonning et al., 1992), but the relative contribution of these two metabolites to the overall oxidation of TAM has not been fully clarified. On the basis of the average in vitro CL_{int} (Table 1), *N*-desmethyl-TAM formation by the high-affinity component was 10.7-fold higher than that of 4-hydroxy-TAM. Assuming that little secondary metabolism occurs during the 10-min incubation used in our study and that the contributions of other metabolic routes are minimal, our data suggest that *N*-desmethyl- and 4-hydroxy-TAM formation accounts for approximately 92 and 7% of primary TAM oxidation, respectively.

The human liver microsomal preparations listed in Table 1 were precharacterized with respect to the activity of individual P450s. For example, the activity of CYP2D6 in the HLMs listed in Table 1 was 135 and 24.5 pmol/min/mg protein in HG23 and HG112, respectively (and undetectable in HG06), whereas that of CYP3A in HG112, HG23, and HG06 was 17,205, 3320, and 3000 pmol/min/mg protein, respectively. We specifically focused on CYP3A and CYP2D6 because there is evidence that CYP3A and CYP2D6, respectively, are important catalysts of TAM *N*-demethylation (Jacolot et al., 1991; Coller et al., 2004) and 4-hydroxylation (Crewe et al., 1997; Dehal and Kupfer, 1997). The relationship between the activity of individual P450s in these HLMs and CL_{int} (Table 1) was tested using linear regression analysis to obtain preliminary information on the P450s catalyzing the low- and high-affinity component. Our data show that the CL_{int} for the formation of 4-hydroxy-TAM by the high-affinity component was significantly related to CYP2D6 activity ($P = 0.0015$), whereas the low-affinity system was predicted by the activity of CYP2B6 ($P = 0.013$) and to some extent by CYP2C19 ($P = 0.053$) and CYP3A ($P = 0.061$). The CL_{int} for *N*-desmethyl-TAM formation by the high-affinity catalyst in the two HLMs (HG23 and HG112) was related with the activity of CYP3A. However, a higher than expected CL_{int} was observed in HG06. Since both expressed CYP3A4 and CYP3A5 catalyze TAM *N*-demethylation at equivalent rate (described below), the discrepancy we observed in HG06 could stem from the fact that a high CYP3A5 level in HG06 might have not been captured by testosterone 6β-hydroxylation, a reaction marker that was used to measure CYP3A activity in these HLMs, but which is known to be preferentially catalyzed by CYP3A4 (Williams et al., 2002). *N*-Desmethyl-TAM formation by the low-affinity system was better predicted by CYP1A2 activity.

The kinetic parameters for the formation of 3-hydroxy-, 4′-hydroxy-, and α-hydroxy-TAM (and apparent values for M-I formation) in HLMs are shown in Table 2. Formation rates versus TAM concentrations were best fit to a single-site binding equation in all cases except those of α-hydroxy-TAM, which was best described by a substrate inhibition model with substrate inhibition constant (K_{si}) of 221 μM. Although all these metabolites were formed when TAM was incubated with the HLMs tested, we were unable to reliably estimate kinetic parameters for the formation of α-hydroxy-TAM in HG06 and HG23 and for the formation of 4′-hydroxy-TAM in HG06.

Correlation of TAM Primary Metabolism with P450s in a Panel of HLMs. The mean ± S.D. apparent formation rates expressed in picomoles per minute per milligram of protein (range) of M-I, α-hydroxy-, 4-hydroxy-, 3-hydroxy-, 4′-hydroxy-, and *N*-desmethyl-TAM from 10 μM TAM in 10 characterized HLMs were 3.1 ± 2.2 (1.1–7.8), 0.8 ± 1.7 (0–5.3), 2.3 ± 0.9 (0.9–4.3), 1.0 ± 0.9 (0–3.1), 0.9 ± 0.9 (0–2.8), and 79.0 ± 34.8 (54.2–165.0), respectively. The formation rate of *N*-desmethyl-TAM in these HLMs was 39.2- ± 19.8-fold (range, 13.8- to 68.4-fold) higher than that of 4-hydroxy-TAM and 10.4- ± 4.2-fold (range, 6.6- to 15.1-fold) higher than the summation of all other TAM primary metabolites. The data regarding formation rates of M-I should be interpreted carefully because the identity of this metabolite was not known. Nevertheless, these results and the kinetic analysis presented in Table 1 suggest that the *N*-demethylation pathway accounts for the majority of TAM oxidation and that the contribution of all other pathways is probably minimal.

The formation rates of α-hydroxy-, 4′-hydroxy-, *N*-desmethyl-TAM, and M-I correlated significantly with the activity of CYP3A ($r = 0.76–0.96$; $P = 0.01–0.0001$) and CYP2B6 ($r = 0.64–0.81$; $P = 0.044–0.004$). The activity of other P450s showed no correlation with any of these metabolites (data not shown). Whether CYP3A and CYP2B6 are both involved in catalyzing these reactions is not apparent from these data because we noted a significant correlation between the activity of CYP3A and CYP2B6 ($r = 0.80$; $P = 0.006$) in the panel of HLMs studied. Inhibition experiments in HLMs and data from expressed P450s (described below) suggest that, except 4′-hydroxy-TAM formation that was cat-

TABLE 2

Kinetic parameters for the metabolism of TAM to α-hydroxy-TAM, 4′-hydroxy-TAM, 3-hydroxy-TAM, and M-I in HLMs

Apparent formation rates (pmol/min/mg of protein) of metabolites against TAM concentrations were fit to a single-site binding equation (4′-hydroxy-TAM, M-I and 3-hydroxy-TAM) or a substrate inhibition equation (α-hydroxy-TAM) as detailed under *Data Analysis*.

Metabolites	HG112			HG23			HG06		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
M-I	11.9	5.2	2.3	2.9	4.5	0.6	7.5	6.5	1.2
α-Hydroxy-TAM	6.5	1.5	4.5	NE	NE	NE	NE	NE	NE
3-Hydroxy-TAM	2.1	4.5	0.5	0.8	5.3	0.2	3.0	5.2	0.6
4′-Hydroxy-TAM	5.0	4.0	1.3	1.9	22.2	0.1	NE	NE	NE

V_{max}, pmol/min/mg of protein; K_m, μM; and V_{max}/K_m, μl/min/mg of protein; NE, not estimated.

alyzed in part by CYP2B6, CYP3A (but not CYP2B6) was the principal catalyst of α-hydroxy-TAM, *N*-desmethyl-TAM, and M-I. Formation rates of 3-hydroxy- and 4-hydroxy-TAM showed no correlation with the activity of any of the P450s tested (data not shown), except a nonsignificant trend for the formation of 4-hydroxy-TAM with the activity of CYP2D6 ($r = 0.56$; $P = 0.09$) and CYP2C9 ($r = 0.45$; $P = 0.20$).

Inhibition of TAM Primary Metabolism in HLMs. The effect of P450 isoform-specific chemical inhibitors on TAM primary metabolism in HLMs ($n = 4$) was tested to identify the P450s involved. The main findings (data not shown) could be summarized as follows. First, ketoconazole (1 μM) and troleandomycin (50 μM) inhibited TAM *N*-demethylation by an average of 62% (range, 28–90%) and 78% (range, 61–90.2%), respectively; sulfaphenazole (20 μM) also showed inhibition in three HLMs by an average of ~31% (range, 24–35%), but it had no effect in one HLM (HG112). Second, quinidine (1 μM) partially inhibited (by an average of 47.7%; range, 32–56%) 4-hydroxy-TAM formation in three HLMs (HK23, HG23, and HG112), whereas this inhibition was minimal (by <12%) in another HLM (HL2); ketoconazole and troleandomycin, although having negligible effect on 4-hydroxy-TAM formation in the three HLMs inhibitable by quinidine, were moderate inhibitors in HL2 (by an average of >45%), suggesting a contribution of CYP3A in this specific HLM. Third, ketoconazole (1 μM) was a potent inhibitor of TAM metabolism to M-I (by 58%), α-hydroxy-TAM (by 77%), and 3-hydroxy-TAM (to an undetectable level). Fourth, thioTEPA (50 μM) and omeprazole (5 μM) decreased 4'- and 3-hydroxy-TAM formation by ~39 and ~26%, respectively. The effect of other inhibitors on TAM primary metabolism was negligible (data not shown).

TAM Primary Metabolism by Expressed Human P450s. To further probe the specific P450 involved, TAM metabolism to its primary metabolites was determined in a panel of expressed P450s. TAM metabolism to M-I was catalyzed at the highest rate by CYP3A4 (with small contribution of CYP3A5, CYP2B6, CYP2C8, and CYP2D6), to α-hydroxy-TAM by CYP3A4 (with contribution of CYP3A5), to 4-hydroxy-TAM by CYP2D6 (other isoforms that showed activity include CYP2C19 > CYP2B6 > CYP2C9 > CYP1A2), to 3-hydroxy-TAM by CYP3A5 (with small activity of CYP2B6 and CYP3A4), to 4'-hydroxy-TAM by CYP2B6 (and CYP2C19), and to *N*-desmethyl-TAM by multiple P450s (CYP2D6 > CYP2C19 > CYP3A5 ≅ CYP3A4 > CYP1A2 = CYP2B6).

Full kinetic analyses were performed for selected P450s that showed significant activity toward formation of TAM primary metabolites. The K_m values obtained by fitting formation rates versus substrate concentrations to a single-site enzyme model were compared with those values obtained in HLMs (Tables 1 and 2). The K_m values derived for the formation of *N*-desmethyl-TAM by CYP2D6, CYP3A4, and CYP3A5 (11.6, 12.6, and 19.4 μM, respectively) were close to the K_m values obtained in HLMs by the high-affinity system. CYP2D6, consistent with previous reports (Crewe et al., 2002), catalyzes TAM *N*-demethylation at the highest rate (CL_{int} for CYP2D6, CYP3A4, and CYP3A5 was 0.33, 0.12, and 0.08 μl/min/pmol P450, respectively), but it appears that CYP3A4 and CYP3A5 are the main catalysts at lower concentrations because no important role could be confirmed for CYP2D6 from the inhibition and correlation data in HLMs. Of note, a marked increase of TAM *N*-demethylation has been reported when healthy volunteers were pretreated with rifampin (Kivisto et al., 1998), a drug known to induce CYP3A and other P450s (but not CYP2D6). In systems that consist of multiple P450s (e.g., HLMs and in vivo in humans), the contribution of CYP2D6 in TAM *N*-demethylation appears to be minimal. Although expressed CYP2B6 shows catalytic activity toward TAM *N*-demethylation, the small inhibitory effect (<20%) of thioTEPA (a relatively selective CYP2B6 inhibitor; Rae et al., 2002) and the high K_m value derived from expressed CYP2B6 (126 μM) makes it unlikely that this isoform represents the high-affinity system. The significant correlation between TAM *N*-demethylation and CYP2B6 activity in a panel of HLMs may stem from a significant correlation between CYP2B6 and CYP3A activity in these HLMs (Ward et al., 2003). Consistent with earlier reports (Crewe et al., 1997, 2002), we found significant activity of CYP2C9, CYP2C19, and CYP1A2 in TAM *N*-demethylation, but the high K_m values derived from CYP2C9 (118 μM) and CYP2C19 (39.4 μM) and the fact that the inhibition of this reaction by specific inhibitors of these enzymes was small in HLMs suggest that these enzymes play a minimal role in TAM *N*-demethylation in vivo.

Although the K_m values for 4-hydroxy-TAM formation by CYP2B6, 2C9, 2C19, 2D6, CYP3A4, and 3A5 were <10 μM (range, 4–9.8 μM), the CL_{int} (microliters per minute per picomole of P450) was relatively higher for CYP2C19 (0.09), CYP2D6 (0.08), and CYP2B6 (0.014) compared with the CL_{int} obtained in CYP3A5, CYP3A4, or CYP2C9 (<0.003) (data not shown). The kinetic parameters for the formation of other TAM primary metabolites were also calculated (data not shown) and essentially confirmed that CYP3A4 was the main enzyme responsible for the metabolism of TAM to M-I and α-hydroxy-TAM, CYP3A5 was the main enzyme responsible for TAM 3-hydroxylation, and CYP2B6 and CYP2C19 were important catalysts of TAM 4'-hydroxylation.

Summary of TAM Primary Metabolism in HLMs and Expressed P450s. Based on the data described above in HLMs and expressed P450s, the proposed human routes of TAM biotransformation to its primary metabolites and the specific P450s catalyzing them are summarized in Fig. 1. In this part of the study, we identified additional TAM primary metabolites. We carried out comprehensive kinetic analyses, which, together with other approaches, allowed the identification (and estimate contribution) of the metabolic pathways and the specific P450s involved at therapeutically relevant concentrations of TAM. *N*-Demethylation of TAM by CYP3A is the major metabolic route of TAM primary metabolism, whereas 4-hydroxy-TAM, which represents minor route, was consistently formed in all HLMs. We also provided the scientific groundwork required for subsequent studies of TAM secondary metabolism.

TAM Secondary Metabolism by HLMs and Expressed P450s

TAM metabolism to its secondary metabolites was studied in HLMs and expressed P450s, using the primary metabolites of TAM, mainly *N*-desmethyl- and 4-hydroxy-TAM, as intermediary substrates. The rationale for selecting these primary metabolites as substrates to characterize secondary metabolism was based on our TAM primary metabolism data described above.

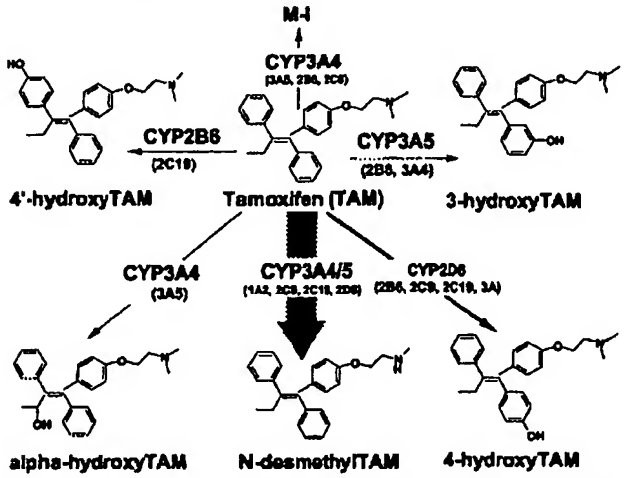

Fig. 1. Proposed in vitro biotransformation pathways of TAM to its primary metabolites and the P450s involved. The relative contribution of each pathway to the overall oxidation of TAM is shown by the thickness of the arrow, and the principal P450 isoforms responsible are highlighted in larger fonts and in bold. M-I, unidentified primary metabolite.

Identification of Secondary Metabolites of TAM in HLMs. Evidence from the literature suggests that TAM undergoes sequential metabolism to several secondary metabolites, including a recently identified active antiestrogen metabolite, endoxifen (Stearns et al., 2003; Johnson et al., 2004). In the present study, we noted secondary metabolites when TAM was incubated with HLMs for 60 min. Here, we tested sequential metabolism of TAM to endoxifen and other secondary metabolites using *N*-desmethyl- and 4-hydroxy-TAM as substrates. Figure 2 shows HPLC traces of human liver microsomal incubations of *N*-desmethyl- and 4-hydroxy-TAM. Three major and two minor (M-II and M-III) metabolite peaks whose formation depended on the P450 system were observed in the microsomal incubations of *N*-desmethyl-TAM (Fig. 2A). The peaks at retention times of 38.2, 24.5, and 14.7 min (Fig. 2A) were consistent with *N*-didesmethyl-TAM, endoxifen, and α-hydroxy *N*-desmethyl-TAM, respectively. α-Hydroxy *N*-desmethyl-TAM was formed from *N*-desmethyl-TAM (Fig. 2A) and TAM (described under *Primary Metabolism*), suggesting that TAM is *N*-demethylated and then α-hydroxylated to α-hydroxy *N*-desmethyl-TAM. Another possible route for the formation of this metabolite could be α-hydroxylation of TAM followed by *N*-demethylation. We tested this possibility by incubating α-hydroxy-TAM in HLMs. Indeed, our data provide evidence that α-hydroxy-TAM undergoes *N*-demethylation to α-hydroxy *N*-desmethyl-TAM (data not shown). The metabolite peaks at 15.5 (M-II) and 25.5 (M-III) min were formed at relatively low rate in the HLMs used (Fig. 2A) under the HPLC conditions tested, and no further attempt was made to characterize their identity. As shown in Fig. 2B, 3,4-dihydroxy-TAM (retention time, ~19.9 min) and endoxifen (retention time, ~25 min) were the main metabolites of 4-hydroxy-TAM. We noted that endoxifen is formed when TAM or *N*-desmethyl- or 4-hydroxy-TAM were used as substrates. Together, our data demonstrate that TAM undergoes sequential metabolism that includes step-wise *N*-demethylation (e.g., to *N*-didesmethyl-TAM), hydroxylation (e.g., to 3,4-hydroxy-TAM), or *N*-demethylation followed by hydroxylation or vice versa (e.g., to endoxifen and α-hydroxy *N*-desmethyl-TAM).

Kinetic Analysis of TAM Secondary Metabolism in HLMs. The kinetics for the metabolism of *N*-desmethyl-TAM to α-hydroxy *N*-desmethyl-TAM, endoxifen, and *N*-didesmethyl-TAM, and of 4-hydroxy-TAM to 3,4-dihydroxy-TAM and endoxifen were determined in different HLMs ($n = 3–4$) that had been characterized for the activity of individual P450 isoforms. Our pilot data indicated that CYP3A and CYP2D6 might be important in TAM secondary metabolism. Thus, HLMs with high and low CYP3A activity and high and low CYP2D6 activity were deliberately selected for the kinetic analyses experiments. The activity of CYP3A (picomoles per minute per milligram of protein) was higher in HG112 (17205) >>> HG23 (3300) > HG06 (3000) ≫ HG93

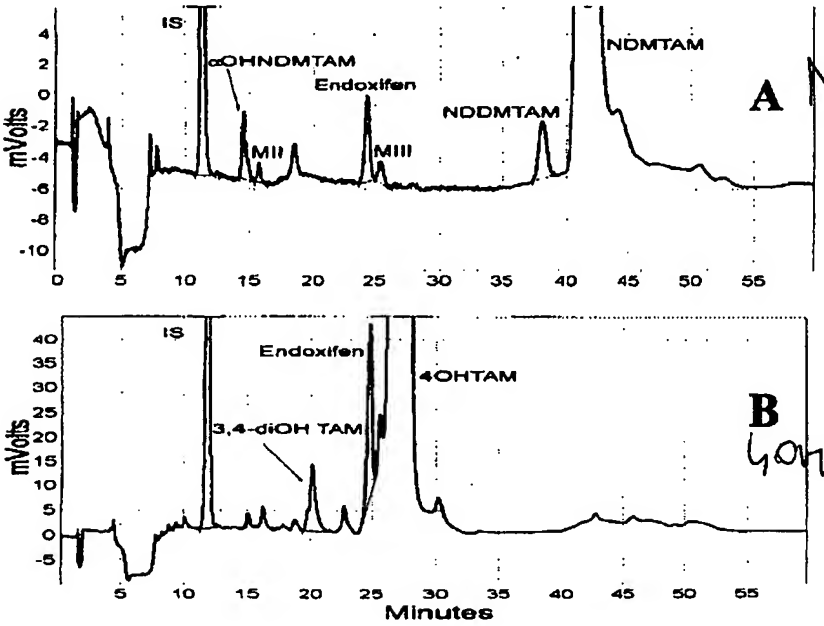

Fig. 2. HPLC traces of human liver microsomal incubate of *N*-desmethyltamoxifen (*N*-DM-TAM) and 4-hydroxytamoxifen (4-OH-TAM). Each substrate (10 μM) was incubated with HLMs (0.1 mg/ml protein) and a NADPH-generating system for 10 min at 37°C and processed as described under *Materials and Methods*. A, metabolite peaks of *N*-DM-TAM. B, metabolite peaks of 4-OH-TAM. NDDM-TAM, *N*-diDM-TAM; endoxifen, 4-hydroxy *N*-DM-TAM; α-OHNDM-TAM, α-hydroxy *N*-DM-TAM; 3,4-diOH-TAM, 3,4-dihydroxy-TAM; M-II and M-III, unidentified metabolites; IS, internal standard (propranolol).

(1660). The activity of CYP2D6 was highest in HG23 (135) >>> HG93 (47) > HG112 (24.5) >>> HG06 (nondetectable). The kinetic parameters for *N*-desmethyl-TAM metabolism are summarized in Table 3. Kinetic parameters for α-hydroxy *N*-desmethyl-TAM formation (Fig. 3A) and endoxifen (Fig. 3B) were obtained by fitting the data to a single-site binding equation. The Eadie-Hofstee plots (data not shown) were characterized by monophasic kinetics. *N*-Didesmethyl-TAM formation on the other hand was best described by biphasic kinetics in two HLMs (HG112 and HG23) (Fig. 3C), with high- (K_{m1} and V_{max1}) and low- (K_{m2} in millimolar range and V_{max2}) affinity components. The in vitro CL_{int} for the high-affinity component was 20- and 3-fold higher than the low-affinity system in HG112 and HG23, respectively. The kinetics of this metabolite in HG06 exhibited monophasic characteristics, and it appears that the high-affinity component is absent in this microsomal preparation (K_m value in HG06 was 22- and 10-fold higher, respectively, than those estimated in HG112 and HG23 for the high-affinity component).

The kinetic parameters for the metabolism of 4-hydroxy-TAM listed in Table 4 were estimated by fitting formation rates versus 4-hydroxy-TAM concentrations to a single-site binding equation 3,4-dihydroxy-TAM and endoxifen in HG06 and HG93) or to a two-site binding equation (endoxifen in HG23, and endoxifen and 3,4-dihydroxy-TAM in HG112). The CL_{int} for endoxifen formation by the high-affinity component in HG112 (71.7 μl/min/mg protein) and HG23 (11.8 μl/min/mg protein), respectively, was 32- and 9.6-fold higher than by the low-affinity component (2.24 and 1.23 μl/min/mg protein in HG112 and HG23, respectively). Similarly, the CL_{int} for the formation of 3,4-dihydroxy-TAM in HG112 by the high-affinity system (59.53 μl/min/mg protein) was 56.9-fold higher than that by the low-affinity system (1.05 μl/min/mg protein).

Representative plots of formation rates of metabolites versus substrate concentrations are shown in Fig. 3 where the data form two HLMs with high and low CYP3A activity (Fig. 3, A, C, D, and E) or high and low CYP2D6 activity (Fig. 3B) are displayed. Visual inspection of these kinetic curves suggest that the metabolism of *N*-desmethyl-TAM to α-hydroxy *N*-desmethyl-TAM (Fig. 3A) and of 4-hydroxy-TAM to 3,4-dihydroxy-TAM and endoxifen (Fig. 3, D and E) is exclusively dependent on CYP3A activity, whereas CYP2D6 appears to be the main enzyme mediating the formation of endoxifen from *N*-desmethyl-TAM (Fig. 3B). The formation of *N*-didesmethyl-TAM was also dependent on CYP3A activity, but to a lesser extent (Fig. 3C). To obtain pilot information as to which isoforms might be involved at therapeutically relevant substrate concentrations, we examined the relationship between the in vitro CL_{int} values obtained for the high-affinity component and the activity of P450 isoforms in the HLMs. The CL_{int} for *N*-desmethyl-TAM metabolism to α-hydroxy *N*-desmethyl-TAM and the CL_{int} for 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM and endoxifen correlated with the activity of CYP3A (data not shown). CYP3A activity in HG112 was 10.4-fold higher than in HG93, and there was a ~14- to 15-fold higher CL_{int} for the metabolism of 4-hydroxy-TAM to its metabolites (Table 4). The formation of endoxifen from *N*-desmethyl-TAM was dependent on CYP2D6. Consistent with the involvement of CYP2D6 in endoxifen formation, HG06 (a phenotypically poor CYP2D6 metabolizer preparation) formed endoxifen at a very low rate (Table 3). The CL_{int} derived for endoxifen in HG23 (5.42 μl/min/mg protein), a microsomal preparation with high CYP2D6 activity, was over 80-fold higher than that obtained from HG06 (0.06 μl/min/mg protein). We observed an increase in K_m value for *N*-didesmethyl-TAM formation with decreasing CYP3A activity (Table 3), suggesting that the higher affinity enzyme responsible for catalyzing this reaction might be CYP3A. Endoxifen is formed when both 4-hydroxy- and *N*-desmethyl-TAM are used as substrates. However, no relationship was observed between the CL_{int} for endoxifen formation from 4-hydroxy-TAM and the CL_{int} for endoxifen formation from *N*-desmethyl-TAM (r^2 = 0.05; P = 0.85), further supporting the involvement of different enzymes in 4-hydroxylation of *N*-desmethyl-TAM and *N*-demethylation of 4-hydroxy-TAM to endoxifen. Together, our data point to an important role of CYP3A in all metabolic routes of *N*-desmethyl- and 4-hydroxy-TAM, except in *N*-desmethyl-TAM 4-hydroxylation, which seems to be dependent on CYP2D6 activity.

Correlation Analysis of TAM Secondary Metabolism in HLMs. The rate of metabolism of *N*-desmethyl- and 4-hydroxy-TAM was determined in a panel of characterized HLMs. The average ± S.D. (range) formation rates of α-hydroxy *N*-desmethyl-TAM, endoxifen, and *N*-didesmethyl-TAM from *N*-desmethyl-TAM (10 μM) in 11 HLMs were 33.5 ± 18.1 (range, 11–74.4), 7.9 ± 5.5 (range, 1.6–18.7), and 27.0 ± 11.7 (12.7–53.5) pmol/min/mg protein, respectively. Formation rates of α-hydroxy *N*-desmethyl-TAM correlated significantly with that of *N*-didesmethyl-TAM (r^2 = 0.63; P = 0.004), but formation rates of endoxifen did not show any correlation with those of α-hydroxy *N*-desmethyl- or *N*-didesmethyl-TAM (r^2 <0.1; P > 0.35). The average ± S.D. (range)

TABLE 3

Kinetic parameters for the metabolism of N-desmethyl-TAM to α-hydroxy *N*-desmethyl-TAM, endoxifen, and *N*-dideamethyl-TAM in HLMs

Since endoxifen was formed very slowly in HG06 (undetectable at substrate concentrations <25 μM), the kinetic parameters estimated from a single-site binding equation were overestimated and are not listed in the table; only mean values from HG23 and HG112 are presented. Data for the formation of *N*-didesmethyl-TAM were characterized by biphasic kinetics in HG23 and HG112 (low and high affinity) and by monophasic kinetics in HG06. The low affinity K_m values were in millimolar ranges, and the kinetic data that represent the high affinity system (V_{max1}, K_{m1}, and V_{max1}/K_{m1}) are presented here.

HLMs	α-Hydroxy *N*-Desmethyl-TAM[a]			Endoxifen[a]			*N*-Didesmethyl-TAM		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
HG06	36.0	6.8	6.2			0.06	203	95.8	2.1
HG23	19.7	5.9	3.4	32.2	5.9	5.4	26.6	8.8	3.0
HG112	94.3	4.7	20.2	6.0	4.6	1.4	62.2	4.3	14.6
Mean	50.0	5.5	9.9	19.1	5.2	3.4	97.3	36.3	6.6
±S.D.	39.2	0.7	9.0			2.5	93.2	51.6	6.9

V_{max}, pmol/min/mg of protein; K_m, μM; and V_{max}/K_m, μl/min/mg of protein.

[a] Formation rates (apparent) vs. substrate concentrations were best fit to a one-site binding equation using a nonlinear regression analysis (see *Data Analysis*).

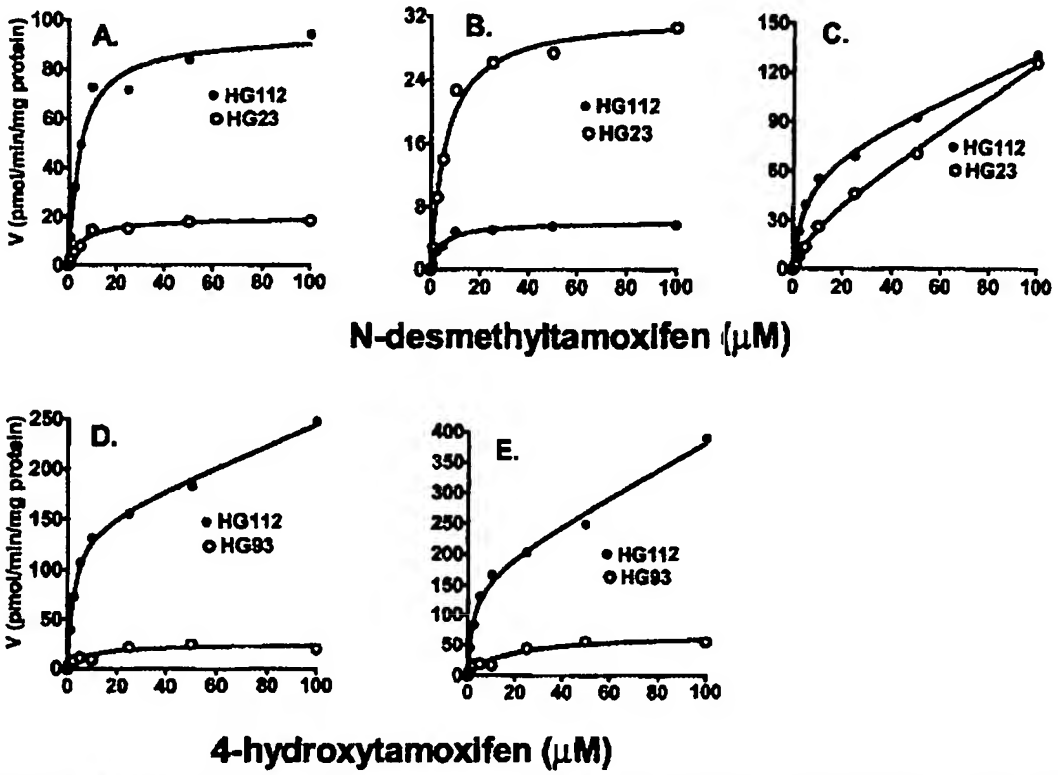

Fig. 3. Representative kinetics for the metabolism of *N*-desmethyltamoxifen (*N*-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) in HLMs. Formation rates (picomoles per minute per milligram of protein) were fit to one- or two-site binding equation as described under *Results* in detail. Upper panel, representative plots of *N*-desmethyl-TAM concentration versus formation rates of α-hydroxy-*N*-desmethyl-TAM (A), endoxifen (B), and *N*-didesmethyl-TAM (C) in two HLMs. Lower panel, representative plots of 4-hydroxy-TAM concentration versus formation rates of 3,4-dihydroxy-TAM (D) and endoxifen (E) in two HLMs. The HLMs selected were characterized in terms of individual P450 activity: CYP3A activity (picomoles per minute per milligram of protein) was high in HG112 (17205), medium in HG23 (3320), and low in HG93 (1660); and CYP2D6 activity (picomoles per minute per milligram of protein) was high in HG23 (135) than in HG112 (24.5). Each point represents average of duplicate incubations.

TABLE 4

Kinetic parameters for the metabolism of 4-hydroxy-TAM to 3,4-dihydroxy-TAM and endoxifen in HLMs

Average (apparent) formation rates vs. substrate concentrations were best fit to a single-site (3,4-dihydroxy-TAM and endoxifen in HG06 and HG93) or two-site binding (endoxifen in HG23 and HG112 and 3,4-dihydroxy-TAM in HG112) equations using a nonlinear regression analysis (see *Data Analysis* and *Results*). Kinetic parameters for the high affinity components (V_{max1}, K_{m1}, and V_{max1}/K_{m1}) are presented here.

HLMs	3,4-Dihydroxy-TAM			Endoxifen		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
HG112	144	2.4	59.5	162	2.3	71.7
HG23	ND	ND	ND	77	6.5	11.8
HG06	41	3.1	13.2	68	8.7	7.8
HG93	27	6.4	4.1	67	14.9	4.5
Mean	70.3	4.0	25.6	93	8.1	23.9
±S.D.	64.0	2.1	29.7	46	5.3	32.0

V_{max}, pmol/min/mg of protein; K_m, μM; and V_{max}/K_m, μl/min/mg of protein.

formation rates of 3,4-dihydroxy-TAM and endoxifen from 4-hydroxy-TAM were 23.4 ± 18.1 (range, 9.3–69.8) and 40.2 ± 29.8 (range, 14.4–112.5) pmol/min/mg protein, respectively. A significant correlation was noted between 3,4-dihydroxy-TAM and endoxifen formation rates in the HLMs tested (r^2 = 0.89; $P < 0.0001$).

Preliminary analyses of the data in Tables 3 and 4 suggested that CYP2D6 and CYP3A might be important to the metabolism of *N*-desmethyl- and 4-hydroxy-TAM. We arbitrarily classified the HLMs as high (106.4 ± 19.7 pmol/min/mg protein; n = 5) and low (20.6 ± 16.2 pmol/min/mg protein; n = 6) CYP2D6 activity HLMs and high (9730 ± 4063 pmol/min/mg protein; n = 5) and low (3123 ± 1045 pmol/min/mg protein; n = 5) CYP3A activity. The rate of endoxifen formation from *N*-desmethyl-TAM in the HLMs with high CYP2D6 activity was significantly higher (12.8 ± 3.8 pmol/min/mg protein) than those in HLMs with low CYP2D6 activity (3.7 ± 1.8 pmol/min/mg protein) (P = 0.003). Formation rates of α-hydroxy *N*-desmethyl-TAM and *N*-didesmethyl-TAM from *N*-desmethyl-TAM, and of 3,4-dihydroxy-TAM and endoxifen from 4-hydroxy-TAM in HLMs with high CYP3A activity were significantly higher (40.3 ± 19.9, 34.5 ± 10.3, 33.7 ± 21.3, and 58.9 ± 33 pmol/min/mg protein, respectively) than those values obtained in HLMs

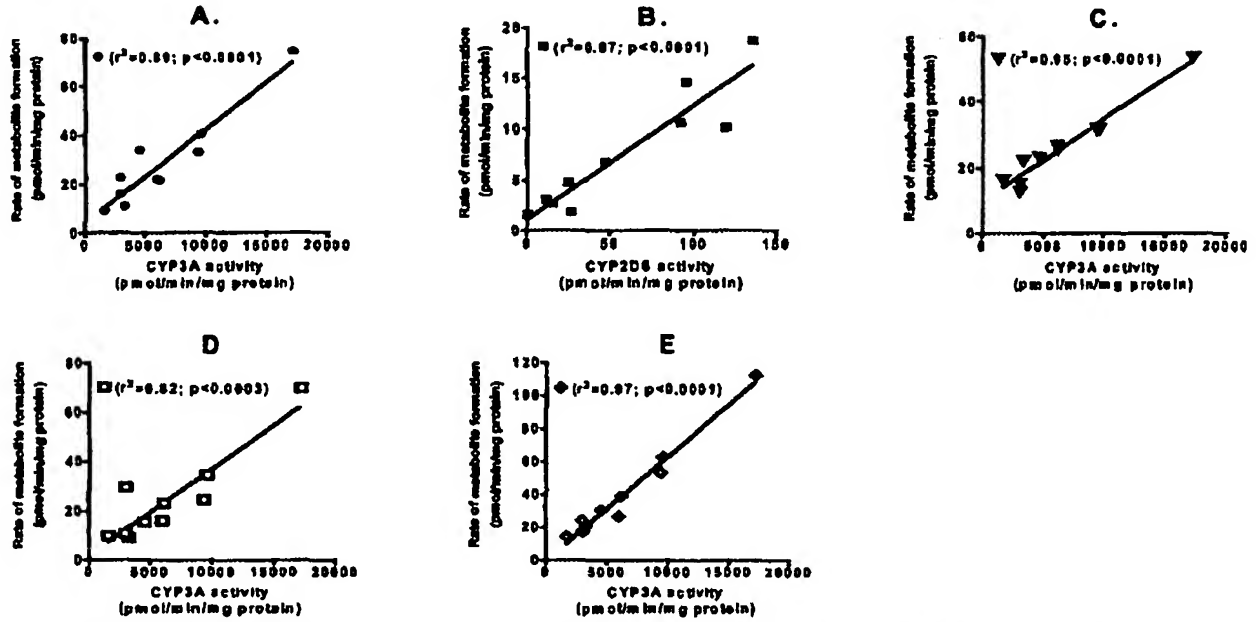

Fig. 4. Correlation between the activity of P450 isoforms and the metabolism of *N*-desmethyltamoxifen (*N*-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) in a panel of different HLMs. Upper panel, correlation between formation rates from *N*-desmethyl-TAM of α-hydroxy *N*-desmethyl-TAM and CYP3A activity (A), endoxifen and CYP2D6 activity (B), and *N*-didesmethyl-TAM and CYP3A activity (C). Lower panel, correlation between formation rates of 3,4-dihydroxy-TAM (D) and of endoxifen (E) from 4-hydroxy-TAM and CYP3A activity. Each point represents average of duplicate incubations.

with low CYP3A activity (25.4 ± 13.2, 17.9 ± 4.6, 15.1 ± 8.6, and 21.5 ± 6.3 pmol/min/mg protein, respectively) ($P < 0.05$).

When correlation analyses were performed across the panel of HLMs tested, CYP3A correlated significantly with rates of *N*-desmethyl-TAM metabolism to α-hydroxy *N*-desmethyl-TAM (Fig. 4A) and *N*-didesmethyl-TAM (Fig. 4C) and 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM (Fig. 4D) and endoxifen (Fig. 4E). There was a statistically significant correlation between endoxifen formation from *N*-desmethyl-TAM and CYP2D6 activity (Fig. 4B). In addition, rates of α-hydroxy *N*-desmethyl-TAM and *N*-didesmethyl-TAM formation correlated significantly with the activity of CYP2B6 (r^2 = 0.58 and 0.61; P = 0.01 and 0.007, respectively) and with total P450 content (r^2 = 0.54 and 0.44; P = 0.016 and 0.038, respectively) (data not shown). Similarly, rates of 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM and endoxifen showed significant correlation with the activity of CYP2B6 (r^2 = 0.49 and 0.61; P = 0.024 and 0.007, respectively). Despite the statistically significant correlation between the activity of CYP2B6 and formation rates of most secondary metabolites, these data alone do not confirm that CYP2B6 is important catalyst of these reactions because, as described above, there is a significant correlation between the activity of CYP2B6 and CYP3A. P450s other than those mentioned here showed no statistically significant correlation with any of the metabolic routes of *N*-desmethyl- and 4-hydroxy-TAM.

Inhibition of TAM Secondary Metabolism in HLMs. The inhibitory effects of isoform-specific inhibitors on the metabolism of *N*-desmethyl- and 4-hydroxy-TAM in HLMs are shown in Fig. 5. Ketoconazole and troleandomycin were the most potent inhibitors of *N*-desmethyl-TAM metabolism to α-hydroxy *N*-desmethyl-TAM (by ~76–92%; Fig. 5A) and

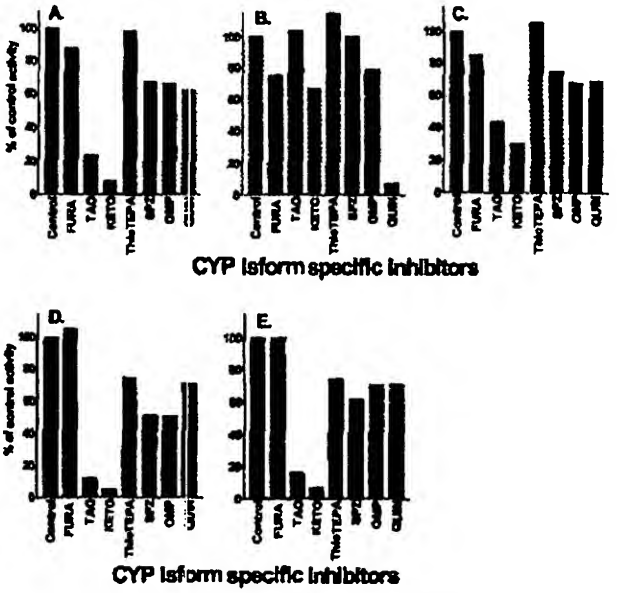

Fig. 5. Inhibition of *N*-desmethyltamoxifen (*N*-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) metabolism by P450 isoform-specific inhibitors in HLMs. The isoform-specific inhibitors used were 20 μM furafylline (FURA, CYP1A2), 50 μM thioTEPA (CYP2B6), 20 μM sulfaphenazole (SPZ, 2C9). 10 μM omeprazole (OMP, 2C19), 1 μM quinidine (QUIN, 2D6), and 1 μM ketoconazole (KETO) and 50 μM troleandomycin (TAO, 3A). Rates of metabolite formation during incubation with the inhibitors are presented as percentage of control (without inhibitor) activity. Upper panel, inhibition of *N*-desmethyl-TAM metabolism in HLMs to α-hydroxy *N*-desmethyl-TAM (A), endoxifen (B), and *N*-didesmethyl-TAM (C). Lower panel, inhibition of 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM (D) and endoxifen (E). Data are average of duplicate incubation measurements.

N-didesmethyl-TAM (by ~56–70%; Fig. 5C) and 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM (by >88%; Fig. 5D) and endoxifen (by >84%; Fig. 5E). The formation of endoxifen from *N*-desmethyl-TAM was almost completely inhibited by quinidine (Fig. 5B). Other inhibitors showed only mild to moderate inhibitory effect on the metabolism of *N*-desmethyl- or 4-hydroxy-TAM.

TAM Secondary Metabolism by Expressed P450 Isoforms. The metabolism of *N*-desmethyl- and 4-hydroxy-TAM by a panel of expressed human P450s is summarized in Fig. 7. *N*-Desmethyl-TAM metabolism to α-hydroxy *N*-desmethyl-TAM was catalyzed at the highest rate by CYP3A4 and CYP2D6, whereas other P450s had less activity (Fig. 6A); to endoxifen by CYP2D6 (Fig. 6B); and to *N*-didesmethyl-TAM by CYP2D6 (Fig. 6C), with participation of CYP3A4, CYP3A5, CYP2C19 CYP2C9, CYP2B6, and CYP1A2. The metabolism of 4-hydroxy-TAM to 3,4-dihydroxy-TAM (Fig. 6D) and endoxifen (Fig. 6E), respectively, was catalyzed by CYP3A5 and CYP2D6 at the highest rates.

Discussion

We report a systematic characterization of TAM sequential metabolism in HLMs and expressed P450s, including complete kinetic analyses. The data obtained may serve as a basis to predict and estimate the contribution of those metabolic pathways and P450s relevant to TAM clearance and its conversion to pharmacologically active metabolites in vivo at therapeutic concentrations.

TAM Biotransformation to Its Primary Metabolites. A thorough quantitative understanding of the primary metabolism of TAM is critical because the rate of primary metabolism determines the clearance of the parent drug, the production of more active antiestrogens (e.g., 4-hydroxy-TAM) or more toxic metabolites (e.g., α-hydroxy-TAM), and the rate at which secondary metabolites that include potent antiestrogens (e.g., endoxifen) or more toxic compounds are formed. We identified six primary metabolites of TAM and the specific P450s involved in metabolism to them (Fig. 1). Our data, consistent with clinical studies (Lonning et al., 1992; Stearns et al., 2003), demonstrate that the liver is an efficient catalyst of *N*-desmethyl-TAM formation, accounting for the majority of TAM oxidation. Our results confirm previous reports (Jacolot et al., 1991; Crewe et al., 1997, 2002) that TAM *N*-demethylation is catalyzed by the CYP3A subfamily and provide the first evidence that both CYP3A4 and CYP3A5 are catalysts of this reaction at low TAM concentrations.

The hydroxylated metabolites, which represent minor routes, are unlikely to play a significant role in TAM clear-

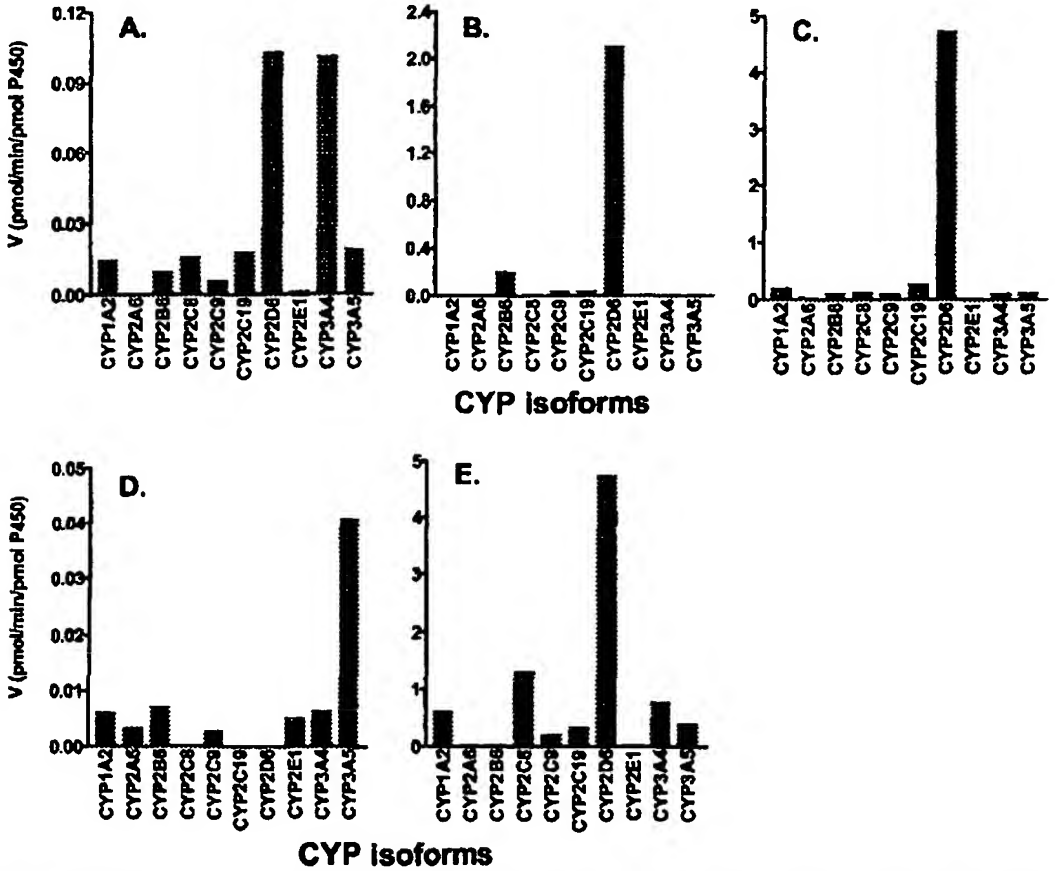

Fig. 6. Metabolism of *N*-desmethyltamoxifen (*N*-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) by a panel of recombinant human P450 isoforms. Upper panel, metabolism of *N*-desmethyl-TAM to α-hydroxy *N*-desmethyl-TAM (A), endoxifen (B), and *N*-didesmethyl-TAM (C). Lower panel, metabolism of 4-hydroxy-TAM to 3,4-dihydroxy-TAM (D) and endoxifen (E). Data are presented as average picomoles product per minute per picomole P450 of duplicate incubation.

ance, but the possibility that these metabolites contribute to its activity/toxicity in vivo cannot be ruled out. TAM 4-hydroxylation is the most studied in this respect because it has been shown that 4-hydroxy-TAM is approximately 30- to 100-fold more potent antiestrogen than TAM (Borgna and Rochefort, 1981; Coezy et al., 1982; Jordan, 1982; Robertson et al., 1982) and because it is believed to be on the path to a reactive intermediate formation that binds covalently to proteins (Dehal and Kupfer, 1999). For these reasons, several investigators have attempted to identify P450s responsible for formation of 4-hydroxy-TAM, with varying results (e.g., Crewe et al., 1997, 2002; Dehal and Kupfer, 1997; Coller et al., 2002). We conducted comprehensive studies in multiple HLMs and expressed P450s. Our data suggest that TAM 4-hydroxylation can be predominantly catalyzed by CYP2D6 in some HLMs with high CYP2D6 activity. However, in conditions with diminished (or absent) CYP2D6 activity, it seems that other isoforms become important. These data are consistent with the involvement of multiple P450s and with our clinical data that show relatively little interpatient variability of 4-hydroxy-TAM in the plasma of breast cancer patients taking tamoxifen (Stearns et al., 2003). α-Hydroxy-TAM and its sulfated metabolites have been implicated in TAM-induced toxicity in vitro and animal studies (White, 2003). The present data and previous reports (White, 2003; Coller et al., 2004) suggest that CYP3A4 and CYP3A5 are important catalysts of TAM α-hydroxylation, raising the possibility that high CYP3A activity in patients may enhance TAM-induced toxicity. We have also provided the first evidence that 4′- and 3-hydroxy-TAM are formed in HLMs and that CYP2B6 (and probably CYP2C19) and CYP3A5, respectively, might be the principal catalysts of these reactions. The relevance of 4′- and 3-hydroxy-TAM to the multiple effects of the drug and their abundance in plasma of patients remains to be determined. Limited studies available in the literature suggest that these metabolites might have higher affinity for the estrogen receptor than TAM (Ruenitz et al., 1982; Roos et al., 1983).

TAM Sequential Biotransformation to Its Secondary Metabolites. We describe here the first comprehensive in vitro characterization of TAM metabolism to its secondary metabolites, using its primary metabolites as intermediaries (Fig. 7). We have demonstrated that *N*-desmethyl-TAM and 4-hydroxy-TAM, like TAM, undergo extensive oxidation by the P450 system to a number of metabolites: α-hydroxy *N*-desmethyl-TAM, endoxifen, and *N*-didesmethyl-TAM were the major *N*-desmethyl-TAM metabolites; and 3,4-dihydroxy-TAM and endoxifen were the principal metabolites of 4-hydroxy-TAM. Our previous (Stearns et al., 2003) and current works have demonstrated that endoxifen is formed primarily from *N*-desmethyl-TAM, but we have shown herein that endoxifen is also formed from 4-hydroxy-TAM.

Several lines of evidence obtained from a variety of experimental approaches clearly show a prominent role of CYP3A in TAM secondary metabolism: we have shown for the first time that the metabolism of *N*-desmethyl-TAM to α-hydroxy *N*-desmethyl-TAM and of 4-hydroxy-TAM to endoxifen is predominantly catalyzed by CYP3A, and we have confirmed CYP3A as the principal catalyst of *N*-desmethyl-TAM metabolism to *N*-didesmethyl-TAM and of 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM, consistent with other reports (Dehal and Kupfer, 1999; Coller et al., 2004). These findings may have important implications. 3,4-Dihydroxy-TAM is probably further oxidized to a reactive intermediate that covalently binds to proteins (Dehal and Kupfer, 1999). *N*-Didesmethyl-TAM appears to be highly concentrated in the liver and is a potent inhibitor of certain P450s that include CYP3A (Comoglio et al., 1996), and this metabolite may mediate drug interactions involving TAM, or it may modify TAM-induced toxicity by inhibiting formation of P450-catalyzed reactive and toxic metabolites of TAM (Comoglio et al., 1996). Little is known regarding α-hydroxy *N*-desmethyl-TAM, but, like α-hydroxy-TAM (White, 2003), it might participate in TAM-induced genotoxicity. It is known that the expression of CYP3A4/5 is highly variable largely due to interindividual differences in the response to various environmental exposures (coadministered drugs, herbal medicines, nutritional supplements) and as the result of genetic polymorphisms that code for CYP3A4/5 proteins. Given the major role played by CYP3A in catalyzing a number of pri-

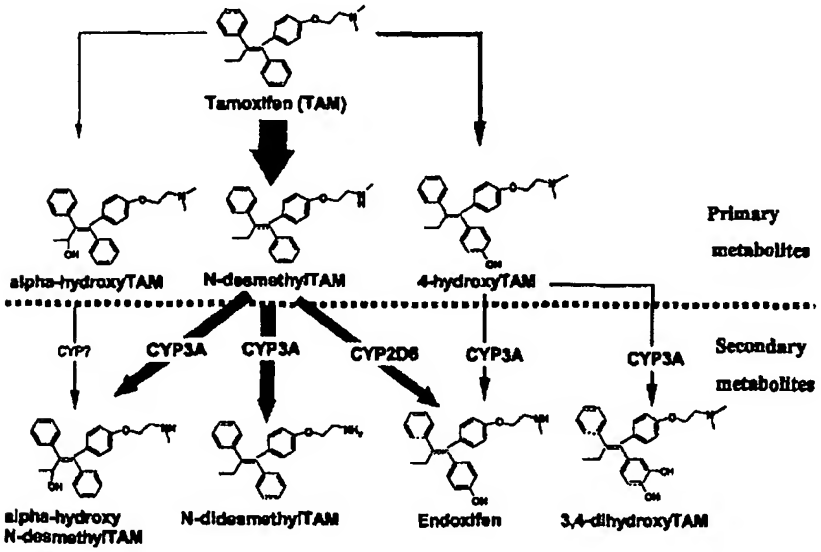

Fig. 7. Proposed in vitro biotransformation pathways of TAM to its secondary metabolites and the P450 isoforms involved. The thickness of each arrow indicates the relative contribution of each pathway to the formation of a specific metabolite. The principal enzymes responsible are highlighted in larger fonts and in bold. The primary metabolism of TAM is described in Fig. 1.

mary and secondary metabolic pathways of TAM, we would expect that interindividual differences in CYP3A activity alters TAM activation/detoxification patterns and its efficacy/toxicity in vivo.

Our recent clinical study showed that the plasma concentrations of endoxifen in breast cancer patients exhibit large interindividual variation (Stearns et al., 2003). Although endoxifen was formed from both *N*-desmethyl- and 4-hydroxy-TAM, the P450s involved in its formation depended on the substrate used: CYP2D6 when *N*-desmethyl-TAM was used as a substrate and CYP3A when 4-hydroxy-TAM was used as a substrate. Despite the efficient conversion of 4-hydroxy-TAM to endoxifen in vitro, the quantitative contribution of this route is likely small. Instead, the 4-hydroxylation of *N*-desmethyl-TAM by CYP2D6 appears to be the major source of endoxifen production in vivo. First, consistent with our in vitro data, we have clinical evidence that endoxifen plasma concentrations in breast cancer patients receiving TAM (20 mg/day) are significantly reduced by coprescription of paroxetine (a known inhibitor of CYP2D6) and in patients who carry variant alleles of CYP2D6 (Stearns et al., 2003). Second, the steady-state plasma *N*-desmethyl-TAM concentrations after therapeutic doses of TAM are much higher (on the average >70-fold) than those of 4-hydroxy-TAM (Lonning et al., 1992; Stearns et al., 2003), essentially in agreement with our in vitro findings that approximately 90% of TAM oxidation is accounted for by *N*-desmethyl-TAM formation. Whether other factors, such as altered production of the precursor (*N*-desmethyl-TAM) from TAM by CYP3A or altered elimination of endoxifen itself due to conjugation by phase II enzymes, contribute to the interpatient variability in endoxifen production in vivo remains to be tested.

Endoxifen is of particular interest to us for a number of reasons. Although this metabolite was identified in human bile in 1988 (Lien et al., 1988) and subsequently in other human biological fluids including plasma (Lien et al., 1989, 1990, 1991), its biological significance was not clear until recently. A series of in vitro studies conducted by our group suggest that endoxifen exhibits similar potency to 4-hydroxy-TAM in terms of its ability to bind with estrogen receptors, in suppressing estrogen-dependent growth of human breast cancer cell lines (Stearns et al., 2003; Johnson et al., 2004), and in global estrogen-dependent gene expression (Lim et al., 2004). There is evidence that endoxifen is much more abundant in plasma of breast cancer patients than 4-hydroxy-TAM (Lien et al., 1990; Stearns et al., 2003). Thus, we speculate that a significant part of TAM pharmacological activity in vivo may be due to the conversion of TAM to active metabolites, most notably endoxifen. The metabolic activity of CYP2D6 exhibits high interindividual variability, mainly due to the polymorphism of the *CYP2D6* gene (over 80 alleles and allele variants have been described, many of which result in loss of enzyme function) (Zanger et al., 2004) but also due to several drugs that potently inhibit the activity of CYP2D6 and mimic the inactivating polymorphisms of the enzyme. It follows that variations in endoxifen concentrations that result from CYP2D6 polymorphisms and drug interactions may influence antitumoral efficacy and side effects of TAM. It is interesting to note that plasma TAM concentrations have been reported to be poor predictors of therapeutic outcome (Bratherton et al., 1984), but that they are also poor predictors of endoxifen concentrations in breast cancer patients (Y. Jin, Z. Desta, V. Stearns, B. Ward, H. Ho, J. Lee, T. Skaar. A. M. Storniolo, L. Li, A. Araba, et al., unpublished data). Further research is warranted to relate endoxifen plasma concentrations with validated clinical outcomes.

In conclusion, we provide a systematic and comprehensive characterization of TAM biotransformation to an array of primary and secondary metabolites. Our data make it clear that TAM metabolism is complex and that multiple approaches as opposed to a single approach are necessary to appropriately identify the metabolic routes of TAM and the P450s responsible at therapeutically relevant concentrations. Overall, CYP3A and CYP2D6 were identified as major enzymes involved in the principal TAM sequential metabolic routes (Figs. 1 and 7). Despite the recent introduction of aromatase inhibitor drugs for the treatment of breast cancer (Baum et al., 2002), TAM remains the endocrine treatment of choice for ER-positive tumors in premenopausal women and as a chemopreventive in women at high risk for the disease. Given that TAM and its metabolites exhibit different pharmacological activities that contribute to the overall therapeutic effect, tissue selectivity, and toxicity, our data should allow improved understanding of the mechanisms and factors that control TAM clearance, activation, and effects in vivo. They also form the basis for a variety of future studies designed to determine the effects of genetic polymorphisms and drug interactions on TAM response and toxicity.

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TAMOXIFEN ANTIESTROGENS. A COMPARISON OF THE ACTIVITY, PHARMACOKINETICS, AND METABOLIC ACTIVATION OF THE *CIS* AND *TRANS* ISOMERS OF TAMOXIFEN

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SUMMARY

In the rat uterus, *cis*-tamoxifen (ICI-47,699, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(*E*)-ene) is a full estrogen agonist, while *trans*-tamoxifen (ICI-46,474, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene is a partial antagonist–partial agonist. In this investigation, we have compared the bioactivity of the tamoxifen isomers in the rat, and utilizing these isomers in tritium-labeled form, we have examined their metabolism and pharmacodynamics. In immature rats, *trans*-tamoxifen is a partial agonist–partial antagonist in terms of uterine weight stimulation, while *cis*-tamoxifen is a pure agonist; both isomers give little stimulation of uterine peroxidase, while only *trans*-tamoxifen is able to antagonize peroxidase stimulation by estradiol. After administration of [^{3}H]-*trans*-tamoxifen to rats *in vivo*, there is a progressive accumulation of *trans*-hydroxytamoxifen and a yet more polar metabolite in the nuclear fraction of the uterus. With [^{3}H]-*cis*-tamoxifen, on the other hand, there is no accumulation of these metabolites in the uterus: in the nuclear fraction, there is a metabolite that is slightly less polar than *cis*-hydroxytamoxifen, and in the cytosol, a metabolite slightly more polar than *cis*-tamoxifen (but different from the nuclear metabolite). There is no evidence for isomerization of the isomers of tamoxifen or hydroxytamoxifen. In *in vitro* incubations with rat liver microsomes, both [^{3}H]-*trans*-tamoxifen and [^{3}H]-*cis*-tamoxifen undergo hydroxylation and to a lesser extent demethylation. Thus, the selective accumulation of *trans*-hydroxytamoxifen in the uterus appears to result from its greater affinity for the estrogen receptor (285%, vs estradiol = 100%) relative to that of *cis*-hydroxytamoxifen (5%). The different metabolites that accumulate in the uterus following treatment with *trans* or *cis* tamoxifen may account for their different agonist antagonist character.

INTRODUCTION

The triphenylethylene antiestrogen tamoxifen has proven to be useful in the treatment of human breast cancer [1, 2] and in the suppression of estrogen-stimulated uterine growth and the growth and development of hormone-dependent mammary tumors in experimental animals [2–4]. However, while tamoxifen (*Z*-tamoxifen; ICI-46,474, "Nolvadex"), which is a *trans* isomer,* is an antagonist of estrogens in the rat, the *cis* isomer of tamoxifen (*E*-tamoxifen; ICI-47,699) is an estrogen agonist [3, 5, 6]. It is now well documented that many antiestrogens, including *trans*-tamoxifen, undergo metabolism to phenolic forms that have enhanced affinity for estrogen receptors [7–15]. These metabolites are selectively accumulated in the nuclear estrogen receptor in target tissues and thus appear to play an important role in the action of these compounds *in vivo*. Therefore, in order to understand the molecular basis for the differing agonist vs antagonist actions of the *cis* and *trans* isomers of tamoxifen, we have prepared these isomeric forms in high specific activity, tritium-labeled form [16]; we have evaluated the interaction of these isomers with target tissues, and we have compared their pharmacokinetics and metabolism. The results presented in this report indicate that there are significant differences in the distribution of the metabolites of the tamoxifen isomers in target tissues, which may contribute to the differences in their bioactivity.

* The stereochemical designations "*cis*" and "*trans*" cannot be applied unambiguously to compounds in the tamoxifen series. In this paper, as is traditional, we have used the two unsubstituted phenyl groups in tamoxifen as reference groups to designate the *cis* and *trans* disposition about the double bond; thus, *trans*-tamoxifen corresponds to the *Z* isomer and *cis*-tamoxifen to the *E* isomer.

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EXPERIMENTAL SECTION

Chemical materials

n-Butyllithium as a solution in hexane and 50% sodium hydride in oil were purchased from Alfa (Ventron); boron triboromide (99.9%) from Apache Chemicals; 5% palladium on carbon from Engelhard,

4 Å molecular sieves from Union Carbide (Linde). *Cis*- and *trans*-tamoxifen and *trans*-hydroxytamoxifen were supplied by ICI Ltd (Macclesfield, U.K.) or by Stuart Pharmaceuticals (ICI, America). Other reagents and solvents were of analytical reagent grade or better and were purchased from Aldrich, Baker, Burdick and Jackson, Fisher, Eastman or Mallinckrodt.

Solvents and reagents were used as purchased, except that tetrahydrofuran (THF) was dried immediately before use by distillation from sodium and benzophenone, and dimethylformamide (DMF) and hexamethylphosphoric triamide (HMPA) were dried by distillation from calcium hydride, purged free of oxygen with argon and then stored over 4 Å molecular sieves.

Instrumentation and chemical procedures

Analytical thin-layer chromatography was performed using 0.20 mm silica gel plastic-backed plates (Pre-Coated TLC Sheets SILICA GEL 60 F254, Merck Cat. 5775) and compounds were visualized by ultraviolet light (254 nm) or iodine vapor. Preparative thin-layer chromatography was carried out using 2 mm glass-backed silica gel plates (Pre-Coated TLC Plates SILICA GEL F-254, Merck). Column chromatography was performed using 0.05–0.20 mm silica gel (Brinkman). Medium-pressure liquid chromatography (MPLC) was performed with a system described previously [17].

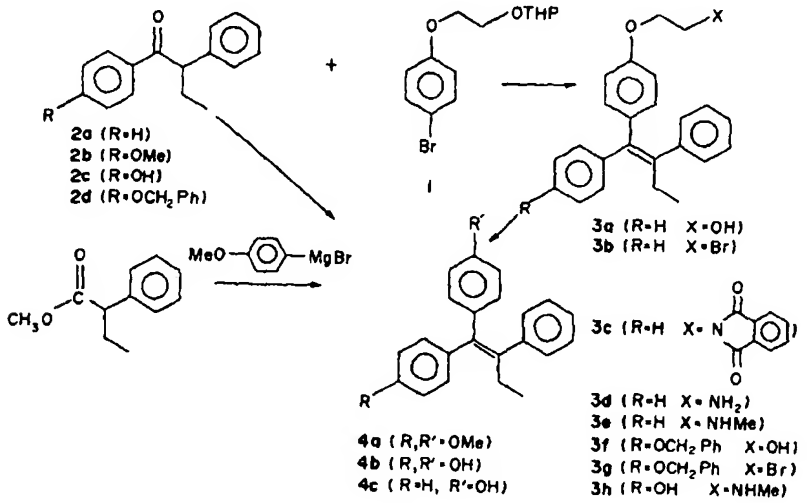
Melting points were determined on a Fisher–Johns Melting Point Apparatus and are not corrected. Infrared spectra were taken as KBr pellets or as carbon tetrachloride, chloroform, or carbon disulfide solutions as indicated parenthetically in the experimental section. Data are presented in cm^{-1} and only the important diagnostic bands are reported. Proton magnetic resonance spectra (^{1}H-n.m.r.) were obtained at 90 MHz on a Varian EM-390 spectrometer and at 220 MHz on a Varian HR-220 spectrometer. Chemical shifts are reported in the delta (δ) system of units relative to tetramethylsilane (TMS) as an internal standard. Routine mass spectra were obtained on a Varian MAT Model CH-5 mass spectrometer; exact mass determinations were obtained on a Varian Model 731 instrument. Microanalytical data were provided by the Microanalytical Service Laboratory of the University of Illinois School of Chemical Sciences.

Except where mentioned otherwise, a standard procedure was used for product isolations; this involved quenching by addition to water, exhaustive extraction with a solvent (washing of extract with aqeuous solutions, on occasion), drying over an anhydrous salt, and evaporation of solvent under reduced pressure. The particular solvents, aqueous washes (if used), and drying agents are mentioned in parentheses after the phrase "product isolation".

Synthetic methods

In the following sections are described the synthesis of tamoxifen derivatives and analogs used in the chromatographic analyses of tamoxifen metabolism. The compounds prepared and their route of synthesis are outlined in Scheme 1.

4-[2-(2-Tetrahydropyranyloxy)ethoxy]phenyl bromide (1). *p*-Toluenesulfonic acid (2 mg) was added to a solution of dihydropyran (0.426 g, 5.06 mmol) and 4-(2-hydroxyethoxy)phenyl bromide (Aldrich) (1.0 g, 4.61 mmol) in ether (50 ml) at 0°C. After warming to room temperature and stirring for 10 h, product isolation (5% potassium carbonate, brine, sodium sulfate) gave 1.38 g (99.4%) of product (1) as a colorless liquid. The material was homogeneous by ^{1}H-n.m.r. and t.l.c.



Scheme 1.

and was used without further purification; attempted distillation caused reversion to starting materials. The compound gave: ^{1}H-n.m.r. ($CDCl_3$) δ 1.25–1.90 (bm, 6, ring CH_2 nonadjacent to O), 3.10–4.00 (*m*, 6, ring CH_2 adjacent to O and $—OCH_2CH_2O$), 4.42 (bs, 1, ring methine), 6.49 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to —OR), 7.02 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to —Br); mass spectrum (70 eV) *m/e* (rel. intensity) 302 (4) M^+, 300 (5) M^+, 200 (2), 198 (2), 174 (5), 172 (5), 129 (38), 101 (5), 85 (100), 73 (27), 67 (14), 57 (13), 43 (23).

Anal. Mol. wt calcd for $C_{13}H_{17}BrO_3$: 300.0361. Found: 300.0366.

1,2-Diphenyl-1-butanone (**2a**). Sodium hydride (3.04 g of a 50% dispersion in oil, 63.6 mmol) was rinsed free of oil with THF (2 × 20 ml) and suspended in 20 ml THF at 0°C. Desoxybenzoin (9.57 g, 48.76 mmol) in 50 ml of THF was added dropwise to the sodium hydride. After the addition was complete the yellow mixture was allowed to warm to room temperature and stirred for 4 h. The enolate solution was cooled to 0°C and ethyl iodide (22.8 g, 146.2 mmol) was added in one portion to the vigorously stirred solution. Sodium iodide precipitated almost immediately and after 0.75 h at 0°C the reaction was quenched by the slow addition of 10% hydrochloric acid (20 ml). Product isolation (ether, water, brine, magnesium sulfate) gave 11.3 g of a light brown oil which was crystallized from methanol to yield the product (**2a**) as white needles (10.06 g, 92%) with m.p. 56–58°C (lit. [18] m.p. 58°C); i.r. (CCl_4) 1690 cm^{-1} (C=O); ^{1}H-n.m.r. ($CDCl_3$) δ 0.90 (*t*, 3, J = 7.5 Hz, $—CH_2CH_3$), 1.67–2.36 (*m*, 2, $—CH_2CH_3$), 4.40 (*t*, 1, J = 7.4 Hz, $—CHCH_2CH_3$), 7.23 (bs, 5, alkyl Ar*H*), 7.31 (*m*, 3, Ar*H meta* and *para* to carbonyl), 7.90 (*m*, 2, Ar*H ortho* to carbonyl); mass spectrum (10 eV) m/e (rel. intensity) 224 (4) M^+, 118 (4), 105 (100), 91 (6).

1,2-Diphenyl-1-[4-(2-hydroxyethoxy)phenyl]but-1-ene (**3a**). *n*-Butyllithium (21.8 mL of a 2.4 M solution in hexane, 52.32 mmol) was added dropwise to a solution of 4-[2-(2-tetrahydropyranyloxy)ethoxy] phenyl bromide (**1**) (15.93 g, 52.90 mmol) in 60 ml of THF at −78°C. After stirring the solution for 0.5 h at −78°C, 1,2-diphenyl-1-butanone (11.7 g, 52.16 mmol) in 40 ml THF was slowly added and the reaction was allowed to warm to room temperature. After stirring for 9 h, product isolation (ether, water, brine, sodium sulfate) gave the tertiary alcohol as a colorless viscous oil.

The crude alcohol was dissolved in methanol (200 ml) containing a few drops of concentrated hydrochloric acid and stirred for 5 h at room temperature. Solvent removal *in vacuo*, product isolation (ether, water, brine, magnesium sulfate), and chromatography (1500 g of silica gel eluted with 45–100% ether in hexane) gave the product as a white solid (14.98 g, 83%) with m.p. 86–95. Recrystallization from hexane gave 10.35 g of flocculent crystals with m.p. 107–110°C which by ^{1}H-n.m.r. was the nearly pure *Z* isomer. (For a discussion of the differences in the ^{1}H-n.m.r. spectra of the *E* and *Z* isomers of substituted triarylethylenes see Refs [19, 20].) A small sample was recrystallized again from hexane giving the pure *Z* isomer (**3a**) as white crystals with m.p. 110.5–111.5°C; i.r. (KBr) 3430 cm^{-1} (—OH); ^{1}H-n.m.r. (CCl_4) δ 0.93 (*t*, 3, J = 7.4 Hz, $—CH_2CH_3$), 1.75 (bs, 1, —O*H*, D_2O exchangeable), 2.45 (*q*, 2, J = 7.4 Hz, $—CH_2CH_3$), 3.78–3.91 (*m*, 4, $—OCH_2CH_2O—$), 6.44 (*d*, 2, J = 8.6 Hz, Ar*H ortho* to —OR), 6.69 (*d*, 2, J = 8.6 Hz, ArH *meta* to —OR), 6.80 (*s*, 5, Ar*H cis* to ArOR), 6.90 (apparent *d*, 5, Ar*H cis* to Et); mass spectrum (70 eV) *m/e* (rel. intensity) 344 (12) M^+, 299 (2), 194 (20), 151 (48), 107 (100), 94 (20).

Anal. Mol wt calcd for $C_{24}H_{24}O_2$: 344.1776. Found: 344.1765.

1-[4-(2-Bromoethoxy)phenyl]-1,2-diphenylbut-1(Z)-ene (**3b**). Triphenylphosphine (270 mg, 1.03 mmol) was added to a stirred solution of carbon tetrabromide (288 mg, 0.868 mmol) and 1,2-diphenyl-1-[4-(2-hydroxyethoxy)phenyl]but-1(*Z*)-ene (**3a**) (273 mg, 0.791 mmol) in 20 ml of methylene chloride at 0°C. After 0.25 h the solvent was removed under reduced pressure and the residue was chromatographed (two 20 × 20 cm preparative t.l.c. plates, 45% ether in hexane, R_f = 0.51) affording the product (**3b**) (313 mg, 97%) as a white solid with m.p. 109–112°C. The analytical sample was prepared by a single recrystallization from hexane giving small white needles with m.p. 111–113°C; ^{1}H-n.m.r. (CCl_4) δ 0.92 (*t*, , J = 7.4 Hz, $—CH_2CH_3$), 2.45 (*q*, 2, J = 7.4 Hz, $—CH_2CH_3$), 3.48 (*t*, 2, J = 6.6 Hz, $—OCH_2CH_2Br$), 4.10 (*t*, 2, J = 6.6 Hz, $—OCH_2CH_2Br$), 6.47 (*d*, 2, J = 8.7 Hz, Ar*H ortho* to —OR), 6.71 (*d*, 2, J = 8.7 Hz, Ar*H meta* to —OR), 7.20 (s, 5, Ar*H cis* to ArOR), 7.32 (apparent *d*, 5, Ar*H cis* to Et); mass spectrum (70 eV) *m/e* (rel. intensity) 408 (98) M^+, 406 (100) M^+, 393 (23), 391 (23), 299 (9), 191 (45), 178 (25), 165 (21), 163 (26), 91 (58), 44 (36).

Anal. Calcd for $C_{24}H_{23}BrO$: C, 70.76; H, 5.69; Br, 19.62. Found: C, 70.45; H, 5.57; Br, 19.68.

1,2-Diphenyl-1-[4-[2-(N-phthalimido)ethoxy]-phenyl]but-1(Z)-ene (**3c**). A stirred mixture of the bromo compound **3b** (1.5 g, 3.68 mmol) and potassium phthalimide (0.820 g, 4.42 mmol) in 30 mL of DMF was heated to 50°C. After 11 h, product isolation (chloroform, water, 0.2 N sodium hydroxide, brine, magnesium sulfate) gave a white solid which was recrystallized from chloroform hexane yielding 1.65 g (95%) of product (**3c**) as white plates with m.p. 151–153°C; i.r. ($CHCl_3$) 1720 cm^{-1} (C=O); ^{1}H-n.m.r. (CCl_4) δ 0.91 (*t*, 3, J = 7.3 Hz, $—CH_2CH_3$), 2.44 (*q*, 2, J = 7.3 Hz, $—CH_2CH_3$), 3.94 4.04 (*m*, 4, $—OCH_2CH_2N—$), 6.47 (*d*, 2, J = 8.6 Hz, Ar*H ortho* to —OR), 6.65 (*d*, 2, J = 8.6 Hz, Ar*H meta* to —OR), 7.05 (s, 5, Ar*H cis* to ArOR), 7.18 (*bm*, 5, Ar*H cis* to Et), 7.66 (*m*, 2, Ar*H meta* to C=O), 7.76 (*m*, 2, Ar*H ortho* to C=O); mass spectrum (70 eV) *m/e* (rel. intensity) 473 (25) M^+, 299 (5), 174 (100), 147 (15), 130 (13), 91 (4), 58 (4).

Anal. Calcd for $C_{32}H_{27}NO_3$: C, 81.16; H, 5.75; N, 2.96. Found: C, 81.46; H, 5.74; N, 2.96.

1-[4-(2-Aminoethoxy)phenyl]-1,2-diphenylbut-1(Z)-ene (N,N-bis(*desmethyl*) tamoxifen, **3d**)

A solution of the phthalimide **3c** (0.800 g, 1.69 mmol) and hydrazine (0.300 g, 9.36 mmol) in ethanol (50 ml) was refluxed for 3.5 h. Product isolation (ether, 0.5 N sodium hydroxide, brine, sodium sulfate) and recrystallization from hexane gave the product (**3d**) (0.545 g, 94%) as white crystals with m.p. 89–90°C; i.r. (KBr) 3480–3400 cm^{-1} (—NH_2); ^{1}H-n.m.r. (CCl_4) δ 0.95 (*t*, 3, J = 7.5 Hz, —CH_2CH_3), 2.53 (*q*, 2, J = 7.5 Hz, —CH_2CH_3), 3.01 (*bm*, 2, —OCH_2CH_2N—), 3.95 (*t*, 2, J = 6 Hz, —OCH_2CH_2N—), 6.62 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to —OR), 6.88 (*d*, 2, J = 8.8 Hz, Ar*H meta* to —OR), 7.36 (*s*, 5, Ar*H cis* to ArOR), 7.42 (*s*, 5, Ar*H cis* to Et); mass spectrum (12 eV) *m/e* (rel. intensity) 343 (100) M^+, 300 (38), 93 (10).

Anal. Calcd for $C_{24}H_{25}NO$: C, 83.93; H, 7.34; N, 4.08. Found: C, 83.79; H, 7.47; N, 4.28.

1,2-Diphenyl-1-[4-(2-methylaminoethoxy)phenyl]but-1[(Z)-ene (*N-Desmethyl-tamoxifen*, **3e**). To a solution of the bromo compound (**3b**) (0.50 g, 1.22 mmol) in 20 ml THF was added methylamine (40% aqueous solution) until the reaction solution became cloudy. The mixture was heated in a pressure bottle at 60°C for 24 h and then cooled. Product isolation (ether, 1% potassium carbonate, brine, sodium sulfate) gave an oil which was crystallized from hexane at −25°C, furnishing the product (**3e**) as white crystals (375 mg, 86%) with m.p. 77–78°C; ^{1}H-n.m.r. ($CDCl_3$) δ 0.92 (*t*, 3, J = 7.4 Hz, —CH_2CH_3), 1.32 (*s*, 1, D_2O exchangeable, N*H*), 2.45 (*s*, 3, $NHCH_3$), 2.45 (*q*, 2, J = 7.4 Hz, —CH_2CH_3), 2.70 (*t*, 2, J = 5.8 Hz, —OCH_2CH_2N—), 3.72 (*t*, 2, J = 5.8 Hz, —OCH_2CH_2N—), 6.55 (*d*, 2, J = 8.78 Hz, Ar*H ortho* to −OR), 6.76 (*d*, 2, J = 8.78 Hz, Ar*H meta* to —OR), 7.08 (*s*, 5, Ar*H cis* to ArOR), 7.22 (*bs*, 5, Ar*H cis* to Et); mass spectrum (70 eV) *m/e* (rel. intensity) 357 (10) M^+, 300 (22), 113 (33), 98 (16), 86 (24), 83 (25), 57 (100), 41 (67).

Anal. Calcd for $C_{25}H_{27}NO$: C, 83.99; H, 7.61; N, 3.92. Found: C, 83.83; H, 7.82; N, 3.85.

1-(4-Methoxyphenyl)-2-phenyl-1-butanone (**2b**). This compound was prepared in 96% yield from 1-(4-methoxyphenyl)-2-phenylethanone [19] according to the ethylation procedure give for 1,2-diphenyl-1-butanone (**2a**). The product (**2b**) was a colorless oil: i.r. (CCl_4) 1680 (C=O), 1265 and 1040 cm^{-1} ($ArOCH_3$); ^{1}H-n.m.r. ($CDCl_3$) δ 0.88 (*t*, 3, J = 7 Hz, —CH_2CH_3), 2.0 (*m*, 2, —$CHCH_2CH_3$), 3.73 (*s*, 3, $ArOCH_3$), 4.37 (*t*, 1, J = 7 Hz, —$CHCH_2CH_3$), 6.78 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to —OCH_3), 7.23 (*s*, 5, Ar*H*), 7.93 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to carbonyl); mass spectrum (10 eV) *m/e* (rel. intensity) 254 (3) M^+, 135 (100), 84 (12).

Anal. Mol wt calcd for $C_{17}H_{18}O_2$: 254.1307. Found: 254.1317.

1-(4-Hydroxyphenyl)-2-phenyl-1-butanone (**2c**). *n*-Butyllithium (14.55 ml of a 2.2 M solution in hexane, 32 mmol) was added dropwise to *n*-butylthiol (3.55 g, 39.4 mmol) in freshly distilled HMPA (10 ml) at room temperature. A rapid evolution of butane was evident and a white precipitate formed. After stirring the mixture for 10 min, 1-(4-methoxyphenyl)-2-phenyl-1-butanone (**2b**) (2.0 g, 7.86 mmol) in HMPA (20 ml) was added in one portion. The hexane was distilled from the reaction, and then the yellow solution was stirred at 140°C for 27 h. After cooling to 25°C, product isolation (ethyl acetate, 3 N hydrochloric acid, water, brine, magnesium sulfate) gave the product as a yellow oil. Chromatography (MPLC, 2.5 × 75 cm silica gel column eluted with 25% ethyl acetate in hexane, R_f = 0.16) gave 1.80 g (95%) of the phenol as a white solid with m.p. 133–135°C. A small portion was recrystallized from methanol/water to give the analytical sample with m.p. 133.5–135°C; i.r. (KBr) 3290 (—OH), 1658 (C=O), 1230 cm^{-1} (C—O); ^{1}H-n.m.r. ($CDCl_3$) δ 0.87 (*t*, 3, J = 6 Hz, —CH_2CH_3), 1.67–2.33 (*m*, 2, —CH_2CH_3), 4.37 (*t*, 1, J = 6 Hz, —$CHCH_2CH_3$), 6.22 (*s*, 1, Ar*OH*, D_2O exchangeable), 6.77 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to −OH), 7.25 (*s*, 5, Ar*H*), 7.87 (*d*, 2, J = 8.8 Hz, Ar*H ortho* to carbonyl); mass spectrum (10 eV) *m/e* (rel. intensity) 240 (4) M^+, 121 (100), 57 (8).

Anal. Calcd for $C_{16}H_{16}O_2$: C, 79.97; H, 6.71. Found: C, 80.13; H, 6.56.

1-(4-Benzyloxyphenyl)-2-phenyl-1-butanone (**2d**). A mixture of 1-(4-hydroxyphenyl)-2-phenyl-1-butanone (**2c**) (1 g, 4.16 mmol), benzyl chloride (2.63 g, 20.77 mmol), sodium iodide (10 mg), and anhydrous potassium carbonate (2.88 g, 20.83 mmol) in DMF (30 ml) was stirred at room temperature for 3.25 h. Product isolation (ether, water, brine, magnesium sulfate) gave a light yellow oil which was chromatographed over 100 g of silica gel (10 to 20% acetate in hexane). The product (**2d**) was a colorless oil (1.347 g, 98%) which solidified on standing to give a white solid with m.p. 75.5–76.5°C; i.r. (CCl_4) 1685 cm^{-1} (C=O); ^{1}H-n.m.r. (CCl_4) δ 0.83 (*t*, 3, J = 7.5 Hz, —CH_2CH_3), 1.42–2.18 (*m*, 2, —CH_2CH_3), 4.02 (*t*, 2, J = 6 Hz, —$CHCH_2CH_3$), 4.17 (*s*, 2, $ArCH_2$—), 6.50 (*d*, 2, J = 8.6 Hz, Ar*H ortho* to —OR), 6.88 (*s*, 5, Ar*H*), 7.00 (*s*, 5, Ar*H*), 7.49 (*d*, 2, J = 8.6 Hz, Ar*H ortho* to carbonyl); mass spectrum (10 eV) *m/e* (rel. intensity) 330 (1) M^+, 211 (100), 91 (43).

Anal. Calcd for $C_{23}H_{22}O_2$: C, 83.60; H, 6.71. Found: C, 83.86; H, 6.77.

1-(4-Benzyloxyphenyl)-1-[4-(2-hydroxyethoxy)-phenyl]-2-phenylbut-1(E,Z)-ene (**3f**). This compound was prepared in 93% yield from 4-[2-(2-tetrahydropyranyloxy)ethoxy]phenyl lithium and 1-(4-benzyloxyphenyl)-2-phenyl-1-butanone (**2d**) exactly as described (*vide supra*) for the nonbenzyloxy-substituted analog (**3a**). The product (**3f**), a mixture of geometrical isomers (1:1), was a highly visious oil which, upon standing, became a white solid with m.p. 90–118°C; ^{1}H-n.m.r. ($CDCl_3$) 0.97 (*t*, 3, J = 7.5 Hz, −CH_2CH_3), 2.57 (*q*, 2, J = 7.5 Hz, —CH_2CH_3), 3.90–4.30 (*m*, 5, —CH_2CH_2OH), 5.05 and 5.20 (each *s*, 2, Ph CH_2O—), 6.68–7.73 (*m*, 13, non benzyl Ar*H*) 7.42 (*s*, 5,

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benzyl Ar*H*); mass spectrum (70 eV) *m/e* (rel. intensity) 450 (99) M^+, 359 (47), 266 (14), 221 (14), 91 (100), 44 (34).

Anal. Mol. wt calcd for $C_{31}H_{30}O_3$: 450.2195. Found: 450.2188.

1-(4-Benzyloxyphenyl)-1-[4-(2-bromoethoxy)phenyl]-2-phenylbut-1(E,Z)-ene (**3g**). This compound was prepared in 96% yield from the alcohol **3f** exactly according to the procedure described for the nonbenzyloxy analog (**3b**). The product (**3g**) was a viscous, colorless oil with ^{1}H-n.m.r. ($CDCl_3$) δ 0.96 (*t*, 3, J = 7.5 Hz, $-CH_2CH_3$), 2.55 (*q*, 2, J = 7.5, $-CH_2CH_3$), 3.6 and 3.72 (each *t*, each 1, J = 6 Hz, $-CH_2CH_2Br$), 4.23 and 4.40 (each *t*, each 1, J = 6 Hz, $-OCH_2CH_2Br$) 5.03 and 5.19 (each *s*, each 1, $PhCH_2O$), 6.67–7.70 (*m*, 13, nonbenzyl Ar*H*), 7.33 (*s*, 5, benzyl Ar*H*); mass spectrum (70 eV) *m/e* (rel. intensity) 514 (40), M^+, 512 (40) M^+, 330 (8), 328 (8), 91 (100).

Anal. Mol wt calcd for $C_{31}H_{29}BrO_2$: 512.1351. Found: 512.1352.

1-(4-Hydroxyphenyl)-1-[4-(2-methylaminoethoxy)-phenyl]-2-phenylbut-1-(E,Z)-ene (*N-Desmethylhydroxytamoxifen*, **3h**). Methylamine (1 ml of a 40% aqueous solution) was added to a solution of the bromo compound **3g** (100 mg, 0.194 mmol) in 1.75 mL THF and the reaction was heated to 75°C in a Reacti-Vial (Pierce) for 8 h. Cooling and product isolation (ether, water, brine, sodium sulfate) gave the amine as a highly viscous, light yellow oil. t.l.c. analysis indicated a single component [benzene–piperidine (9:1); R_f = 0.29].

The crude product was dissolved in 4 ml ethanol, 50 mg of 5% palladium on carbon was added, and the mixture was stirred for 6 h under 1 atmosphere of hydrogen. The catalyst was then separated by filtration through Celite, the solvent was removed *in vacuo*, and the residue was purified by preparative t.l.c. [chloroform–triethylamine–methanol (9:1:1)]. The product was obtained as a light yellow foam with ^{1}H-n.m.r. ($CDCl_3$) δ 0.90 (*t*, 3, J = 7 Hz, $-CH_2CH_3$), 2.46 and 2.53 (*s*, each 1.5, $-NHCH_3$ of two isomers), 2.99 and 3.10 (*bs*, each 1, $-CH_2NHCH_3$), 3.91 and 4.09 (*bs*, each 1, $-OCH_2-$), 6.43–7.14 (*m*, 13, Ar*H*); mass spectrum (10 eV) *m/e* (rel. intensity) 373 (78) M^+, 316 (100), 301 (14), 86 (22), 60 (5), 58 (5), 44 (80).

Anal. Mol. wt calcd for $C_{25}H_{27}NO_2$: 373.2004. Found: 373.2023.

1,1-Bis(4-methoxyphenyl)-2-phenylbut-1-ene (**4a**). The methyl ester of 2-phenylbutanoic acid (Aldrich) was prepared by standard procedures, and a solution of the ester (0.89 g, 5 mmol) in 20 ml ether was added dropwise to 4-methoxyphenylmagnesium iodide [prepared from 4-methoxyphenyl iodide (3.5 g, 15 mmol) and magnesium (0.37 g, 15.2 mg-atom] in 100 ml ether. The reaction was stirred overnight at room temperature and quenched with saturated ammonium chloride. Product isolation (ether, water, brine, magnesium sulfate) gave the crude alcohol as a brown oil.

The alcohol was dissolved in pyridine (50 ml) and phosphorous oxychloride (1.68 g, 10.9 mmol) was added dropwise. After stirring overnight, product isolation (ether, 2 N hydrochloric acid, water, brine, magnesium sulfate), and chromatography [MPLC, 2.5 × 75 cm silica gel column eluted with ether–methylene chloride–hexane (1:2:17)] produced the triarylbutene (**4a**) as a white solid (1.49 g, 87%) with m.p. 123–125°C; i.r. ($CHCl_3$) 1255 and 1042 cm^{-1} ($ArOCH_3$); ^{1}H-n.m.r. ($CDCl_3$) δ 0.92 (*t*, 3, J = 7.5 Hz, $-CH_2CH_3$), 2.46 (*q*, 2, J = 7.5 Hz, $-CH_2CH_3$), 3.61 (*s*, 3, $ArOCH_3$), 3.77 (*s*, 3, $ArOCH_3$), 6.42–7.17 (*m*, 8, disubstituted Ar*H*), 7.08 (*s*, 5, monosubstituted Ar*H*); mass spectrum (10 eV) *m/e* (rel. intensity) 344 (100) M^+, 329 (21), 214 (22), 206 (10), 147 (36), 105 (27), 91 (17).

Anal. Calcd for $C_{24}H_{24}O_2$: C, 83.69; H, 7.02. Found: C, 83.49; H, 7.00.

1,1-Bis(4-hydroxyphenyl)-2-phenylbut-1-ene (**4b**). 1,1-*Bis*(4-methoxyphenyl)-2-phenylbut-1-ene (**4a**) (100 mg, 0.290 mmol) was dissolved in methylene chloride (10 ml), and the solution was cooled to −78°C. *Via* gas-tight syringe boron tribromide (0.73 g, 2.19 mmol) was added dropwise to produce a light red solution which was allowed to warm slowly to room temperature. After stirring at room temperature for 4.5 h, the reaction was cooled to −78°C and quenched by the slow dropwise addition of anhydrous methanol (15 ml). Removal of solvents *in vacuo*, product isolation (ethyl acetate, water, brine, magnesium sulfate), and recrystallation from ethanol–water produced the bisphenol **4b** as white, flocculent needles (79 mg, 86%) with m.p. 207–207.5°C; i.r. (KBr) 3432 cm^{-1} (—OH); ^{1}H-n.m.r. (acetone-d_6) δ 0.87 (*t*, 3, J = 7 Hz, $-CH_2CH_3$), 2.37 (*q*, 2, J = 7 Hz, $-CH_2CH_3$), 6.13–7.26 (*m*, 8, disubstituted Ar*H*), 6.8 (*s*, 5, monosubstituted Ar*H*); mass spectrum (10 eV) *m/e* (rel. Intensity) 316 (100) M^+, 301 (15), 222 (10), 199 (35), 183 (15), 94 (18).

Anal. Mol wt calcd for $C_{22}H_{20}O_2$: 316.1463. Found: 316.1462.

1,2-Diphenyl-1-(4-hydroxyphenyl)but-1(E,Z)-ene (**4c**). *n*-Butyllithium (1.92 ml of a 2.4 M solution in hexane, 4.61 mmol) was added dropwise to a solution of 4-(2-tetrahydropyranyloxy)phenyl bromide [21] (1.20 g, 4.67 mmol) in 10 ml THF at −78°C and stirred for 0.5 h. 1,2-Diphenyl-1-butanone (**2a**) (1.0 g, 4.45 mmol) in 10 ml THF was then added and the reaction was allowed to slowly warm to room temperature and stirred for 5.5 h. Product isolation (ether, water, brine, sodium sulfate) gave the alcohol as a colorless oil.

The crude alcohol was dissolved in 30 ml methanol containing a few drops of concentrated hydrochloric acid and the solution was stirred over-night. Product isolation (ether, water, brine, magnesium sulfate) and chromatography (MPLC, 2.5 × 75 cm silica gel column eluted with a 10–25% ethyl acetate in hexane gradient) gave 1.02 g (76%) of **4c** as a white solid as an approximately 1:1 mixture of geometrical isomers as judged by t.l.c. analysis (silica gel, benzene–piperidine

(9:1), more mobile isomer $R_f = 0.29$, less mobile isomer $R_f = 0.22$). The mixture displayed: m.p. 106–112°C (lit. [22] m.p. 104–105°C); i.r. (KBr) 3430 cm^{-1} (—OH); ^{1}H-n.m.r. (CCl_4) δ 0.93 (*dt*, 3, J = 7.26 Hz, $—CH_2CH_3$), 2.46 (*dq*, 2, J = 7.26 Hz, $—CH_2CH_3$), 4.23 and 4.45 (s, altogether 1, $-OH$ of the two isomers, D_2O exchangeable), 6.36 (*d*, 1, J = 8.6 Hz, Ar*H ortho* to —OH in *trans* isomer), 6.64 (*d*, 1, J = 8.6 Hz, Ar*H meta* to —OH in *trans* isomer), 6.68–7.27 (*m*, 12, Ar*H*); mass spectrum (10 eV) *m/e* (rel. intensity) 300 (79) M^+, 285 (10), 94 (46), 42 (100).

A sample of the pure *Z* isomer was obtained by treating 1-[4-(2-bromoethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene (**3b**) (200 mg, 0.491 mmol) with an excess of sodium cyanide (500 mg) in DMF (15 ml) for 10 h at room temperature. Product isolation (ether, water, magnesium sulfate) and recrystallization from hexane gave the product as flocculent crystals with m.p. 125–127°C. The compound was the pure *Z* isomer as judged by ^{1}H-n.m.r. analysis [19] and by t.l.c. corresponded to the more mobile isomer in the *E,Z*-mixture.

Anal. Calcd for $C_{22}H_{20}O$: C, 87.96; H, 6.71. Found: C, 87.75; H, 6.68.

cis-*Hydroxytamoxifen.* A small sample for binding assay was obtained as follows: a sample of pure *trans*-monohydroxytamoxifen was dissolved in a minimum amount of freshly distilled THF (no BHT) and allowed to stand for two days to insure complete *trans–cis* equilibration. The sample was subjected to h.p.l.c. using a Varian model 5060 liquid chromatograph equipped with a Rheodyne 7125 injector, a Beckman LC-75 detector operated at 275 nm, and a LDC Excalibar C_6 (5 μm) spherisorb column (4.6 mm i.d. × 25 cm). The column was eluted with methanol–water–diethylamine (60:39.6:0.4) at 1.5 ml/min, giving clean separation of *trans*-hydroxytamoxifen (identified by co-injection with pure *trans*) and *cis*-hydroxytamoxifen, with the retention times being 10.3 and 14.2 min, respectively. The *cis* fraction from several injections was collected in THF containing 0.025% BHT to prevent isomerization, and the solvents were removed under reduced pressure. The white residue was found to be 98% *cis*-hydroxytamoxifen contaminated by 2% of the *trans* isomer as determined by peak area analysis of a portion that was rechromatographed.

Preparation of radiolabeled tamoxifen isomers. The tritiated tamoxifen isomers were prepared in our laboratories by catalytic hydrogenation of a brominated precursor (2-(4-bromophenyl)-1-[4-(2-dimethylaminoethoxy)phenyl]-1-phenylbut-1(*E,Z*)-ene, using carrier-free tritium gas. The hydrogenation product was purified and the tamoxifen isomers were separated by preparative thin layer chromatography on silica gel, utilizing benzene:triethylamine (9:1, v/v). Each isomer had a radio-chemical purity in excess of 97%, with specific activities of: [^{3}H]-*trans*-tamoxifen—12.2 Ci/mmol and [^{3}H]-*cis*-tamoxifen—6.5 Ci/mmol. Synthesis of the precursor, tritium labeling, and purification will be described in full elsewhere [16].

Biochemical materials

Immature female rats, 20–24 days old (Holtzman; Madison, Wisconsin) were used in this study. The 17β-[6,7-^{3}H]-estradiol (48–54 Ci/mmol) was from New England Nuclear Corporation. The charcoal-dextran slurry contained 5% acid-washed Norit A (Sigma) and 0.5% dextran C (Schwarz–Mann) in 0.01 M Tris, 0.02% sodium azide, pH 7.4 at 25°C. The xylene-based and toluene-based scintillation fluids were those described previously [14].

Biochemical procedures

Preparation of uterine cytosol and competitive binding assays. Cytosol from immature rat uteri (180,000 *g*, 60 min supernatant) was prepared in iced 0.01 M Tris–HCl-0.0015 M EDTA-0.02% sodium azide pH 7.4 at 25°C (TEA buffer). Aliquots of uterine cytosol were incubated with 10^{-8} M [^{3}H]-estradiol and 10^{-10} to 10^{-4} M cold competitor for 16 h at 0°C and were analyzed exactly as described previously [23]. The relative binding ability (RBA) of the competitors is taken as the ratio of the concentrations of unlabeled estradiol/competitor required to inhibit half of the specific [^{3}H]-estradiol binding with the affinity of estradiol set at 100%.

Determination of uterotrophic and antiuterotrophic activity of tamoxifens. Stock solutions of estrogen or antiestrogen were prepared in absolute ethanol immediately before use and were then diluted with sesame oil so that the desired dosage of drug would be contained in the 0.1–0.2 ml of 10% ethanol in oil that was administered. Groups of immature rats (20 days of age) received s.c. injections containing various amounts of *cis*- or *trans*-tamoxifen and/or 1 μg E2 for 3 days and were killed 24 h after the last injection. The uteri were excised and weighed, and then further assayed for peroxidase activity as described below.

Peroxidase assays. Uteri were homogenized (25 mg/ml) in iced 0.01 M Tris–HCl buffer, pH 7.2 and the homogenate was centrifuged at 40,000 *g* for 30 min. Uterine peroxidase was solubilized, as described by Lyttle and DeSombre[24] and by us previously [11], by rehomogenizing the 40,000 *g* × 30 min sediment in 10 mM Tris–HCl, pH 7.2 buffer containing 0.5 M $CaCl_2$. Peroxidase activity was determined exactly as described [11]. The assay mixture, 3.0 ml total volume, contained 13 mM guaiacol, 0.3 mM H_2O_2 and generally 0.3 ml of sample extract. The rate of oxidation of guaiacol was linear with protein concentration of the extract and was measured in the presence of H_2O_2 as indicated by a change in absorbance at 470 nm.

Analysis of the metabolites of the [^{3}H]-tamoxifen isomers generated in vivo. Rats received [^{3}H]-*cis*- or [^{3}H]-*trans*-tamoxifen (5 μg) injected subcutaneously in 0.5 ml of 0.15 M NaCl (saline) containing 6% ethanol. At the indicated times after injection, uteri were

excised, rinsed, and homogenized in iced TEA buffer (6 uteri/2 ml). The homogenate was centrifuged at 800 *g* for 20 min at 4°C, and the nuclear pellet washed 3 times with 3 ml iced TEA buffer and then extracted with 3 ml of ethanol. The 800 *g* supernatant was centrifuged at 180,000 *g* for 60 min at 0°C and the cytosol preparation was extracted with five volumes of ethyl acetate. At the indicated times after injection, serum was prepared and extracted with ethyl acetate [14]. The following extraction efficiencies were obtained: nuclear pellet: at all times >95%; cytosol: 6 h–96% (*cis* and *trans*); 16 h—98% (*cis*), 74% (*trans*); 48 h—87% (*cis*), 57% (*trans*); serum: 6 h—70–85% (*cis* and *trans*); 16 h—75–85% (*cis*), 30–60% (*trans*); 48 h—70–90% (*cis*), 30–50% (*trans*).

Extracts were analyzed by thin layer chromatography on plastic-backed silica gel plates (SILICA GEL 60 F254, Merck No. 5775), as described previously [14] and in the figure legends, using two solvent systems: benzene–triethylamine (9:1, v/v; system A) for optimum resolution of the tamoxifen isomers, and benzene-piperidine (9:1, v/v; system B) for optimum resolution of the hydroxytamoxifen isomers. Standard reference compounds (cf. Table 1) were applied to each chromatogram along with the radiolabeled extract; the relative mobility of the standards were measured under ultraviolet illumination.

Analysis of the metabolites of the [^{3}H]-tamoxifen isomers produced by liver microsomes. Liver microsomes were prepared from immature (day 20–24 day) female rats exactly as described previously [15]. The [^{3}H]-*trans*- or [^{3}H]-*cis*-tamoxifen isomer (10 μCi of each) was incubated with 1.5 ml washed microsomes (88 mg/ml microsomal protein) in a phosphte buffer containing an NADPH regenerating system for periods up to 30 min at 37°C with agitation [15], followed by cooling to 0°C and pelleting the microsomes at 100,000 *g* for 1 h. The pellet, containing 98% of the radioactivity, was resuspended in phosphate buffer and extracted with ethanol, to yield a clear supernatant containing 97% of the radioactivity from the microsomal pellet. The extracts were analyzed by thin layer chromatography according to the methods described above.

RESULTS

Comparative bioactivities of tamoxifen isomers: uterine weight and peroxidase

In immature female rats, *trans*-tamoxifen is a partial estrogen agonist in terms of uterine weight gain (Fig. 1), but is also capable of antagonizing uterine growth stimulated by estradiol. Effective antagonism of the uterine growth stimulated by 1 μg of estradiol is achieved with the 20- and 100-μg dosages of *trans*-tamoxifen. As has been seen previously with other triphenylethylene antiestrogens [25], tamoxifen can antagonize estrogen-stimulated weight gain only down to the level of its own inherent estrogenicity. In contrast to the *trans* isomer, *cis*-tamoxifen is a full

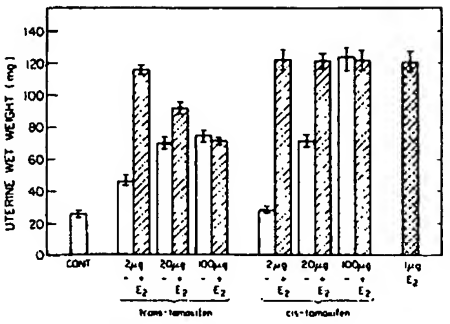


Fig. 1. The effect of the administration of *trans*- or *cis*-tamoxifen alone (open bars), or estradiol plus *trans*- or *cis*-tamoxifen (hatched bars) on uterine wet weight. Estradiol (1 μg) or tamoxifen isomers (2–100 μg), or both compounds together, were injected s.c. in oil once daily at 24 h intervals for three days, and uterine wet weight was determined 24 h following the third injection. The control (cont, oil vehicle) uterine weights are shown in the leftmost bar and the estradiol alone weights are shown in the rightmost bar. Values represent the mean ± SEM of eight immature rat uteri.

estrogen agonist, effecting a uterine weight gain at the 100 μg level equivalent to that of the maximally stimulatory estradiol dose (1 μg). In addition, there is no antagonism of the estrogen-stimulated weight gain by *cis*-tamoxifen.

Peroxidase is an enzyme whose activity has been shown to be markedly increased by estrogen in growth-responsive target tissues [26]. Hence, we examined the effects of the tamoxifen isomers on uterine peroxidase activity (Fig. 2), employing the same dosage regimens as in Fig. 1. Peroxidase activity in

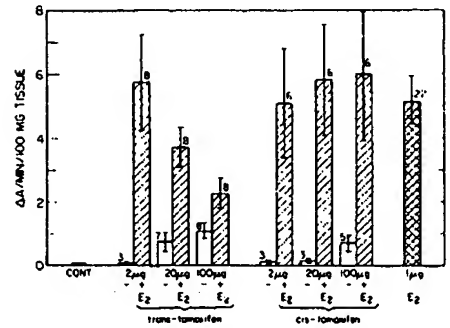


Fig. 2. The effect of the administration of *trans*- or *cis*-tamoxifen alone (open bars), estradiol plus tamoxifen isomers (hatched bars), or estradiol alone (cross hatched bar) on uterine peroxidase activity. Estradiol (1 μg) or tamoxifen isomers (2–100 μg), or both compounds, were injected s.c. in oil once daily at 24 h intervals for three days and peroxidase activity was determined in uteri excised at 24 h following the third injection. Peroxidase activity was nearly undetectable in control uteri (cont, oil vehicle-injected animals). Values represent the mean ± SEM of values from the number of experiments indicated above each bar. Each experiment employed two rat uteri and triplicate determinations.

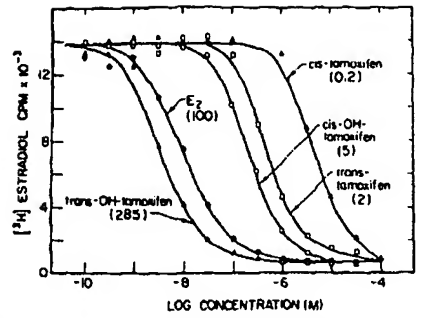


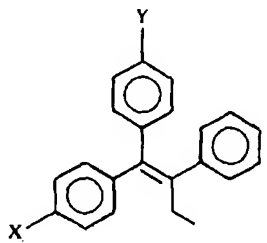
Fig. 3. Competitive binding assay of tamoxifen and hydroxytamoxifen isomers. Uterine cytosol was incubated for 16 h at 0–4°C with the stated concentration of competitor (10^{-10}–10^{-4} M) and 1×10^{-8} M [^{3}H]-estradiol. After incubation, charcoal-dextran (15% by volume, 15 min at 0–4°C) was added to adsorb unbound ligand and radioactivity was determined in the supernatant. The numbers in parentheses indicate the relative binding affinity of each compound for receptor, where estradiol is set at 100.

unstimulated uterus is virtually undetectable and is dramatically increased by estradiol. In contrast, both tamoxifen isomers alone are very weak stimulators of uterine peroxidase, achieving levels only 15–20% that of estradiol at the 100 μg dose. The isomers differ, however, in terms of their ability to antagonize estrogen-stimulated uterine peroxidase, with only *trans* tamoxifen achieving significant antagonism.

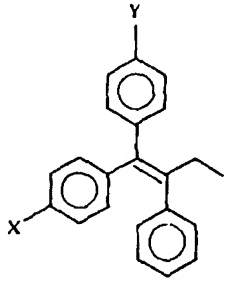
Binding affinity of the tamoxifen isomers to the uterine estrogen receptor

The binding affinity of the (non-radiolabeled) tamoxifen and hydroxy-tamoxifen isomers for the rat uterine estrogen receptor was determined by competitive binding analysis. Comparison of the concentration of these compounds needed to effect a 50% decrease in the specific binding of [^{3}H]-estradiol (Fig. 3 and Table 1) indicates that *trans*-tamoxifen has an affinity 2% that of estradiol; *cis*-tamoxifen has an affinity 0.2% that of estradiol. *trans*-Hydroxytamoxifen, known to be a metabolite of tamoxifen, has an affinity 285% that of estradiol, while *cis*-hydroxytamoxifen has an affinity of only 5%. These results demonstrate the dramatic increase in receptor affinity that occurs upon hydroxylation of the tamoxifen isomers, and they indicate that the trans isomers have a much higher receptor binding affinity than the corresponding *cis* isomers, with this geometric dependence being even greater for the hydroxytamoxifen isomers. The binding affinity of several other tamoxifen analogs (and potential metabolites) are also shown in Table 1; they are discussed in the Discussion Section.

Table 1. Binding affinities of tamoxifen isomers and analogs for rat uterine estrogen receptor*



trans (Z)



cis (E)

X	Y	Compound name (abbreviation)	RBA† *trans* (Z)	RBA† *cis* (E)
1. H	O—CH_2—CH_2—NMe_2	Tamoxifen (T)	2	0.2
2. HO	O—CH_2—CH_2—NMe_2	Hydroxytamoxifen (OH-T)	285	5
3. H	O—CH_2—CH_2—NHMe	*N*-desmethyltamoxifen (desMe-T)	3	—
4. HO	O—CH_2—CH_2—NHMe	*N*-desmethyl-hydroxytamoxifen (desMe-OH-T)	(143)‡	
5. H	O—CH_2—CH_2—NH_2	*N,N*-bisdesmethyl-tamoxifen	2	—
6. H	OH	"Monophenol" (OH)	(95)‡	
7. HO	OH	"Bisphenol" $(OH)_2$	160§	

* Competitive binding assay employed cytosol from immature rat uteri, 10 nM [^{3}H]-estradiol. 10^{-10}–10^{-4} M unlabeled competitor and conditions exactly as described in the legend of Fig. 3.

† The relative binding affinity (RBA) is determined from the molar concentrations of each compound needed to inhibit 50% of the estrogen specific binding; the relative binding affinity of estradiol is set at 100.

‡ Numbers in parentheses represent relative binding affinity values determined on *cis–trans* isomer mixtures (c. 1:1) of the compound indicated.

§ Due to its symmetrical substitution, this compound does not have geometric isomers.

Comparative metabolism of tamoxifen isomers in vivo

In order to compare the metabolism and the uterine interaction of the tamoxifen isomers and their metabolites, immature female rats were given 5 μg doses of *cis* and *trans*-[^{3}H]-tamoxifen (88 μCi and 164 μCi, respectively). Extracts from serum and uterine cytosol and nuclear fractions were obtained at 6,16 and 48 h after injection, and these fractions were analyzed by thin layer chromatography in two solvent systems. System A, benzene-triethylamine (9:1), provided good separation of *cis* and *trans*-tamoxifen, but did not separate the isomeric hydroxytamoxifens, while benzene–piperidine (9:1, system B), gives good separation of the isomeric hydroxytamoxifens and the more polar materials, but it does not separate the two tamoxifen isomers. In addition, some potential metabolites that lack the basic side chain (c.f. Table 1) were included in the chromatographic analysis: the isomeric monophenols (designated **OH**), which would result from side-chain cleavage of the respective isomeric tamoxifens, and a single bisphenol (**(OH)$_2$**), which would result from side chain cleavage of either isomeric monohydroxytamoxifen.

The most striking contrasts were noted in the uterine nuclear fraction. It is evident from Fig. 4, Panels A-C, showing chromatograms run in system B, that with time there is a progressive conversion of *trans*-tamoxifen first into *trans*-hydroxytamoxifen (the predominent form at 16 h) and eventually into a yet more polar metabolite(s) that has chromatographic mobility that is in most cases very similar to that of the bis-phenol (**(OH)$_2$**) in this solvent system, but may not be identical with it (see below). From the insets in these panels, which are portions of chromatograms of the same fractions run in system A, it is clear that there is no interconversion of the tamoxifen isomers. It is apparent from these inset chromatograms, as well, that there is no significant material that co-chromatographs with the monophenol (**OH**).

Chromatographic analysis of uterine nuclear extracts in system B, following *cis*-tamoxifen administration (Fig. 4, Panels D-F), indicates at all times a predominance of *cis*-tamoxifen. There is, however, a progressive accumulation of a metabolite that is slightly more polar than *cis*-tamoxifen, but which migrates clearly ahead of the hydroxytamoxifens. Although this material has not been identified as yet, its chromatographic mobility is consistent with that expected for *N*-desmethyl *cis*-tamoxifen [8]. Again, as seen in the insets, there is no conversion of *cis*-tamoxifen to the trans isomer.

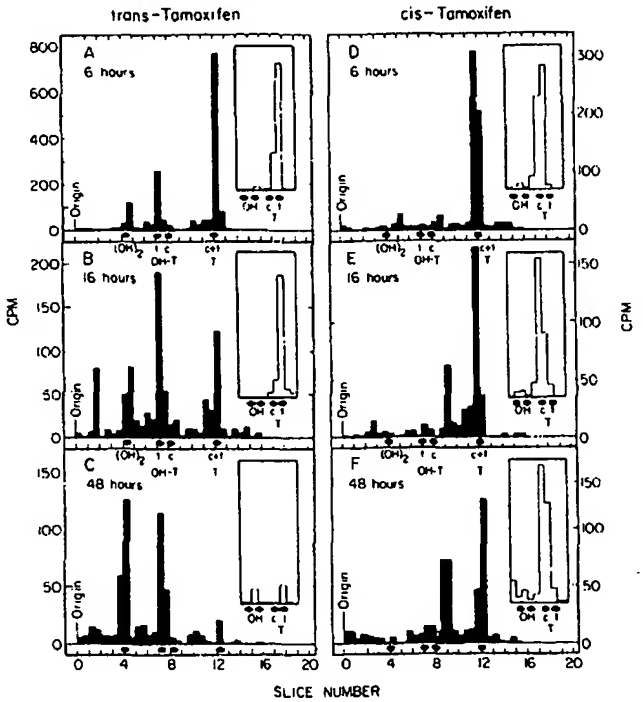


Fig. 4. Chromatographic analysis of the metabolites of the tamoxifen isomers in uterine nuclear extracts. Immature rats (six per group) were injected s.c. with [^{3}H]-*trans*-tamoxifen (5 μg; 165 μCi) or [^{3}H]-*cis*-tamoxifen (5 μg; 88 μCi) and at 6, 16 or 48 h after injection, ethanol extracts of uterine nuclear fractions were prepared as described in Methods, and analyzed by thin layer chromatography in benzene piperidine (9:1, v/v; system B). The panel insets represent a portion of chromatograms developed in a benzene–triethylamine (9:1, v/v) solvent system (system A). Standard compounds were incorporated into each chromatogram, and their mobilities are indicated below the radioactivity profile. Abbreviations and structures for the standards can be found in Table 1.

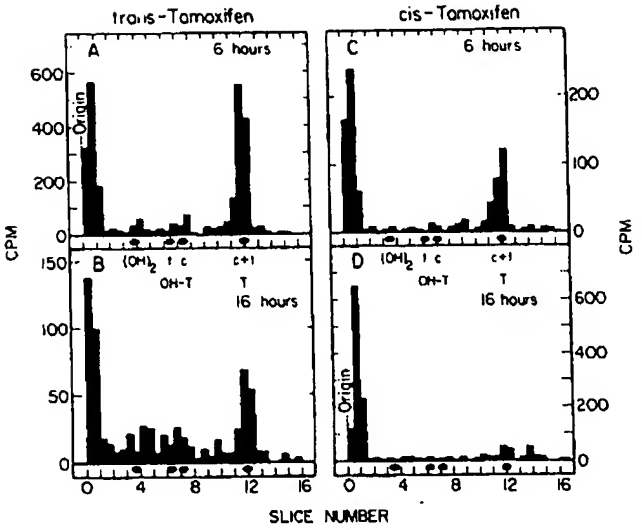


Fig. 5. Chromatographic analysis of the metabolites of the tamoxifen isomers in serum. Rats were injected with the [^{3}H]-tamoxifen isomers as indicated in Fig. 4 legend and at 6 and 16 h after injection, serum was extracted with ethyl acetate. The extracts were analyzed by thin layer chromatography in the benzene–piperidine (9:1, v/v) system (system B).

Analysis of ethyl acetate extracts of serum following administration of *cis* or *trans*-tamoxifen reveals fewer differences between the isomers (Fig. 5). At 6 and 16 h, the predominant forms are either the originally-administered isomer or polar material that migrates very near the origin. There is perhaps a hint of hydroxylated form in the case of *trans*-tamoxifen, but clearly this metabolite is far more prominent in the uterine nuclear fraction (cf. Fig. 4). The serum extractable counts at 48 h were very low (data not shown), too low to give meaningful chromatograms.

Ethyl acetate extracts of uterine cytosol from animals treated with [^{3}H]-*trans*-tamoxifen gave chromatographic profiles very similar to those of serum (data not shown). Chromatograms of extracts of uterine cytosol from animals receiving [^{3}H-*cis*-tamoxifen, however, did show a progressive accumulation of a more polar metabolite (Fig. 6). *cis*-Tamoxifen predominates at 6 and 16 h, but the metabolite is a major form at 48 h.

In several experiments, we have compared the mobility of the cytosolic metabolite of *cis*-tamoxifen with the more polar metabolite of *cis*-tamoxifen that is evident in the nuclear extracts (cf. Fig. 4F). The cytosolic metabolite is clearly less polar than the nuclear metabolite: In system A (Fig. 6C), it migrates just behind *cis*-tamoxifen, with a mobility close to that of the monophenols (OH); there is no activity in this region in the nuclear extracts (cf. Fig. 4, insets in Panels E and F). In system B, in which the nuclear metabolite is clearly resolved from *cis*-tamoxifen (Fig. 4F), the cytosolic metabolite co-migrates with *cis*-tamoxifen (data not shown).

Although the more polar metabolite(s) of *trans*-tamoxifen that accumulates in the uterine nuclear

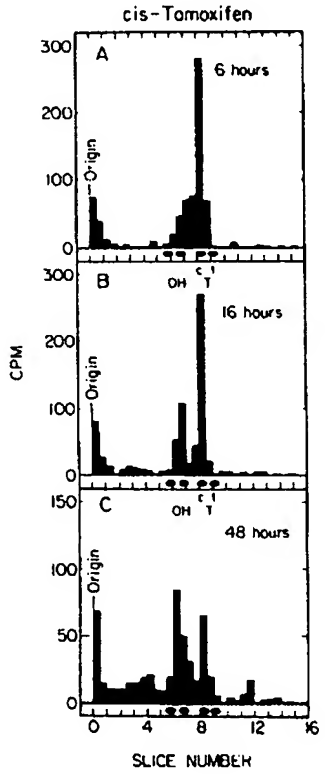


Fig. 6. Chromatographic analysis of the metabolites of *cis*-tamoxifen in uterine cytosol. Immature rats were injected with [^{3}H]-*cis*-tamoxifen as indicated in Fig. 4 legend and at 6, 16 and 48 h after injection ethyl acetate extracts of uterine cytosol preparations were prepared as described in Methods. The extracts were analyzed in the benzene–triethylamine (9:1, v/v) system (system A).

fraction has a chromatographic mobility very similar to that of the bis-phenol $(OH)_2$, it is evident from a close inspection of Panel A of Fig. 4, that all of the polar activity does not migrate with the bis-phenol in every case. Furthermore, from studies that we will describe in detail elsewhere, we believe that this more polar activity may actually be a mixture of several compounds.

Metabolism of tamoxifen isomers by liver microsomes

In order to investigate the relative rates at which the metabolites of the tamoxifen isomers are being produced, we have examined metabolite production *in vitro*, in incubations with rat liver microsomes. Washed microsomes from immature rat liver were incubated at 37°C with the tamoxifen isomers, together with an NADPH regenerating system. The metabolites were obtained by ethanol extraction after various time periods and were analyzed by thin layer chromatography in the systems described above, *N*-desmethyltamoxifen and *N*-desmethylhydroxytamoxifen were included as additional chromatographic standards. The chromatographic pattern of metabolites obtained after 20 min incubation is shown in Fig. 7; the patterns obtained after 10 and 30 min are not substantially different and are not shown.

There are a number of striking features of these data. First, of major significance is the fact that both tamoxifen isomers appear to be producing the same types of metabolites, which by chromatographic analysis appear to be the hydroxytamoxifens, as well as

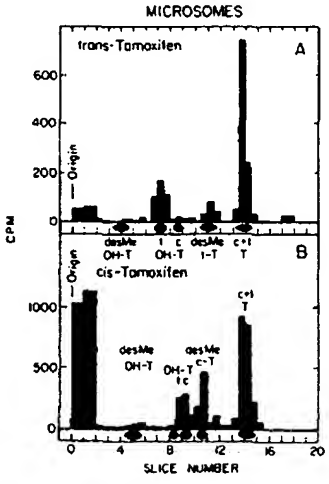


Fig. 7. Chromatographic analysis of the metabolites of the tamoxifen isomers following incubation with rat liver microsomes. [^{3}H]-*trans*- or [^{3}H]-*cis-tamoxifen* (10 μCi) was incubated with rat liver microsomes (13.2 mg protein in 1.5 ml) for 20 min at 37°C, as described in Methods. Ethanol extracts were then prepared and analyzed by thin layer chromatography in benzene–piperidine (9:1, v/v; system B). Standard compounds were incorporated into each chromatogram, and their mobilities are indicated below the radioactivity profile. Abbreviations and structures for the standards can be found in Table 1.

the *N*-desmethyltamoxifens. One noteable difference between the isomers is that much more very polar material (that stays near the origin) is produced in the incubation with *cis*-tamoxifen.

DISCUSSION

It has been an interesting observation that the two isomers of tamoxifen have different activities in the rat: the *trans* isomer being a partial antagonist and partial agonist, but the *cis* isomer being a complete agonist [3, 5, 6]. In this investigation, we have confirmed these differences in activity, and, in addition, we have shown that these isomers differ in their ability to antagonize estrogen-stimulated uterine peroxidase. These findings are of particular interest because at doses at which these compounds are maximally effective as uterotrophic (*cis* isomer) or antiuterotrophic (*trans* isomer) agents, both isomers are poor stimulators of uterine peroxidase activity. Thus, there is an apparent dissociation between uterine growth stimulation and stimulation of uterine peroxidase activity with these triphenylethylenes.

In this study, we have also utilized high specific activity tritium-labeled tamoxifen isomers to examine the comparative metabolism of these compounds in the rat *in vivo*, in order to see whether any of the differences in the activity of these isomers might be due to differences in their pattern of metabolite production. The most striking observation is that polar metabolites accumulate in the uterine nucleus following administration of [^{3}H]-*trans*-tamoxifen. We have previously reported that polar metabolites of the antiestrogens CI628 and U23469 selectively accumulate in the uterine nuclear fraction [13–15, 27, 28]. In the case of *trans*-tamoxifen studied here, the first formed and less polar of these metabolites has been identified by Borgna and Rochefort[8] as *trans*-hydroxytamoxifen and has been shown to be associated with the estrogen receptor [8]; we have not yet identified the nature of the more polar metabolite.

After administration of [^{3}H]-*cis*-tamoxifen, on the other hand, there is no accumulation of the corresponding *cis*-metabolites. Instead, a small amount of a metabolite with polarity between that of *cis*-tamoxifen and *cis*-hydroxytamoxifen appears. The identity of this material has not yet been established, and while its mobility is consistent with that of *N*-desmethyl-*cis*-tamoxifen, its substantial accumulation in the nuclear receptor fraction suggests that its affinity for receptor must be greater than that of *N*-desmethyl-*cis*-tamoxifen. In addition, while there is little accumulation of metabolites from *trans*-tamoxifen in the uterine cytosol fraction, yet another metabolite from *cis*-tamoxifen is evident in this fraction; this material is distinct from the *cis*-tamoxifen metabolite that appears in the nuclear fraction.

From these results alone, it is not clear whether the greater nuclear accumulation of the polar *trans*-tamoxifen metabolites is due to the fact that the

corresponding compounds are not produced from *cis*-tamoxifen or whether the cis isomers of these metabolites (produced from *cis*-tamoxifen) simply have lower affinity for the estrogen receptor. Our further studies indicate that the latter situation appears to be the case.

From the *in vitro* incubations with rat liver microsomes, it appears that both *N*-demethylation and hydroxylation are taking place, and a comparable rates, with both *trans* and *cis*-tamoxifen. Therefore, one may expect that *in vivo*, the corresponding metabolites from *cis*- and *trans*-tamoxifen would be made available by hepatic metabolism at roughly comparable rates. However, from competitive binding assays, it is clear that there are very large differences in the estrogen receptor binding affinities between certain of the isomeric metabolites; the ratio can be as high as 50:1. Thus, the fact that certain metabolites from *trans*-tamoxifen are found to accumulate selectively in the uterine nuclear fraction may simply reflect the fact that only the *trans* isomer of these metabolites has sufficient affinity for the estrogen receptor to be retained in the uterus.

From an analysis of the binding data of these and other potential tamoxifen metabolites (Table 1), it is evident that the process of hydroxylation leads to compounds with substantially increased affinity for the receptor. In contrast, *N*-demethylation has only a slight, if any, effect on receptor affinity, and it is probably for this reason that little *N*-desmethyltamoxifen is found in the uterus following administration of *trans*-tamoxifen. It is of note that *N*-desmethyl-*trans*-tamoxifen is found in the serum of humans on tamoxifen therapy [29].

The monophenols and the bisphenol are potential metabolites of tamoxifen and hydroxtamoxifen, respectively, that would result from cleavage of the basic side chain. They have very high affinity for the estrogen receptor, and from work we have done (D. W. Robertson, B. S. Katzenellenbogen and J. A. Katzenellenbogen, unpublished), they are pure estrogens. Therefore, their formation and their accumulation in the uterine estrogen receptor fraction would be expected to result in an estrogenic effect. In none of the chromatograms from the uterine nuclear fraction was there evidence for the monophenols; the more polar metabolite(s) from *trans*-tamoxifen, however, has mobility that is quite consistent with that of the bisphenol, and further investigation of the identity of this activity is underway. A metabolite of *cis*-tamoxifen that is found in the cytosol has a mobility quite comparable to that of the monophenols. The lack of nuclear accumulation of this metabolite, however, suggests that it does not have substantial affinity for the receptor.

In this study, we endeavored to elucidate whether the differing activities of *cis* and *trans*-tamoxifen in the rat were due to differences in the metabolism of these compounds in the rat. From our studies, we can conclude that there are very significant differences in the pattern of metabolites of *cis* and *trans*-tamoxifen found in the uterus and that these differences appear to result in large part from differences in the affinity of the *cis* and *trans* isomers of certain major metabolites for the estrogen receptor.

In addition, we have found two metabolites of *cis*-tamoxifen that appear to be very different from those derived from *trans*-tamoxifen. One metabolite, in particular, that is only slightly less polar than *cis*-tamoxifen, is accumulated selectively in the uterine nuclear fraction. It is possible that this compound may be a contributor to the agonistic action of *cis*-tamoxifen in the rat.

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Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors α and β

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ABSTRACT

The rat estrogen receptor (ER) exists as two subtypes, ERα and ERβ, which differ in the C-terminal ligand binding domain and in the N-terminal transactivation domain. In this study we investigated the messenger RNA expression of both ER subtypes in rat tissues by RT-PCR and compared the ligand binding specificity of the ER subtypes.

Saturation ligand binding analysis of *in vitro* synthesized human ERα and rat ERβ protein revealed a single binding component for 16α-iodo-17β-estradiol with high affinity [dissociation constant (K_d) = 0.1 nM for ERα protein and 0.4 nM for ERβ protein]. Most estrogenic substances or estrogenic antagonists compete with 16α-[^{125}I]iodo-17β-estradiol for binding to both ER subtypes in a very similar preference and degree; that is, diethylstilbestrol > hexestrol > dienestrol > 4-OH-tamoxifen > *17β-estradiol* > coumestrol, ICI-164384 > estrone, 17α-estradiol > nafoxidine, moxestrol > clomifene > estriol, 4-OH-estradiol > tamoxifen, 2-OH-estradiol, 5-androstene-3β,17β-diol, genistein for the ERα protein and dienestrol > 4-OH-tamoxifen > diethylstilbestrol > hexestrol > coumestrol, ICI-164384 > *17β-estradiol* > estrone, genistein > estriol > nafoxidine, 5-androstene-3β,17β-diol > 17α-estradiol, clomifene, 2-OH-estradiol > 4-OH-estradiol, tamoxifen, moxestrol for the ERβ protein. The rat tissue distribution and/or the relative level of ERα and ERβ expression seems to be quite different, *i.e.* moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal for ERα and prostate, ovary, lung, bladder, brain, uterus, and testis for ERβ. The described differences between the ER subtypes in relative ligand binding affinity and tissue distribution could contribute to the selective action of ER agonists and antagonists in different tissues. (*Endocrinology* 138: 863–870, 1997)

ESTROGENS INFLUENCE the growth, differentiation and functioning of many target tissues. These include tissues of the male and female reproductive systems such as mammary gland, uterus, ovary, testis, and prostate. Estrogens also play an important role in bone maintenance and in the cardiovascular system, where estrogens have certain cardioprotective effects (1). Estrogens are mainly produced in the ovaries and testis. They diffuse in and out of all cells, but are retained with high affinity and specificity in target cells by an intranuclear binding protein, termed the estrogen receptor (ER). Once bound by estrogens, the ER undergoes a conformational change, allowing the receptor to bind with high affinity to chromatin and to modulate transcription of target genes (2). Steroid hormone receptors consist of a hypervariable N-terminal domain that contributes to the transactivation function; a highly conserved central domain responsible for specific DNA binding, dimerization, and nuclear localization, and a C-terminal domain involved in ligand binding and ligand-dependent transactivation function (1). The rat ER cDNA was cloned from uterus and found to be highly homologous to the ER complementary DNAs (cDNAs) cloned from mouse, human, and chicken (3). We recently cloned a novel rat ER cDNA from prostate (4), which we suggested be named rat ERβ subtype to distinguish it from the previously cloned ER cDNA (consequently ERα subtype). The rat ERβ cDNA encodes a protein of 485 amino acid residues with a calculated mol wt of 54200. Rat ERβ protein is highly homologous to rat ERα protein, particularly in the DNA binding domain (> 90% amino acid identity) and in the C-terminal ligand binding domain (LBD) (55%). Saturation ligand binding experiments with *in vitro* synthesized ERβ protein revealed a single binding component for 17β-estradiol (E_2) with high affinity [dissociation constant (K_d) = 0.6 nM]. Expression of ERβ was investigated by *in situ* hybridization, and prominent expression was found in rat prostate (secretory epithelial cells) and ovary (granulosa cells). In cotransfection experiments of Chinese hamster ovary (CHO) cells with an ERβ expression vector and an estrogen-regulated reporter gene, maximal stimulation of reporter gene activity was found during incubation with 1 nM E_2 (4).

The biological significance of the existence of two ER subtypes is at this moment unclear. Perhaps the existence of two ER subtypes provides, at least in part, an explanation for the selective actions of estrogens in different target tissues (5). In fact, the high degree of interspecies conservation of the individual ER subtypes throughout vertebrate evolution (Ref. 6 and our unpublished observations) could suggest that the basis for the selective effects of estrogens resides in the control of different subsets of estrogen-responsive promoters by

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the two ER subtypes. This would implicate differential expression of the ER subtypes in target tissues.

The overall homology between the rat ERα protein LBD and rat ERβ protein LBD is not more than 55% (Fig. 1). Interestingly, the ERβ protein LBD encompassing amino acid residues 223–457 has a low homolgy with the ERα protein LBD between amino acid residues 344–403, whereas outside this stretch the homology is considerably higher (amino acid residues 223–343 and 404–457). The structural core of the LBD of the human ERα protein has recently been mapped by restricted proteolysis, and only one single region within this core was found to be easily accessible to proteases (7). This surface-exposed protease accessible region (human ERα LBD amino acid residues 465–468) is in the center of the stretch showing lowest homology with ERβ protein. The amino acid sequence stretches of the ERβ LBD between amino acids 223–343 and 404–457 are probably, similarly to the highly homologous stretches in the ERα LBD, part of a compact hydrophobic (non-surface-exposed) entity directly contacting the ligand. Although several parts of these stretches are completely conserved, and the amino acid alterations often are conservative, it is possible that interesting differences in ligand binding affinity and/or specificity exist between the ER subtypes. Chemically quite diverse compounds (estrogens, some androgens, phytoestrogens, antiestrogens, and environmental estrogens) have been shown in the past to have estrogenizing activity and to interact with the ER from rat uterus and human breast tumor cells (Ref. 8 and references therein).

In the present study we investigated the ligand binding specificity of the two ER subtypes and the transcript tissue distribution in the adult rat.

Materials and Methods

Materials

The radioligand 16α-[^{125}I]iodo-E_2 ([^{125}I]E_2) was obtained from New England Nuclear (Boston, MA). The unlabeled steroids E_2, 17α-estradiol, estrone, estriol, dehydroepiandrosterone, 5α-dihydrotestosterone, testosterone, progesterone, corticosterone, moxestrol (11β-methoxy-17α-ethynyl-1,3,5(10)-estratrien-3,17β-diol), 4-hydroxy-estradiol (1,3,5(10)-estratriene-3,4,17β-triol), 2-hydroxy-estradiol (1,3,5(10)-estratriene-2,3,17β-triol), 5-androstenediol (5-androstene-3β,17β-diol), 4-androstenediol (4-androstene-3β,17β-diol), 3α-androstanediol (5α-androstane-3α,17β-diol), 3β-androstanediol (5α-androstane-3β,17β-diol), 5α-androstanedione (5α-androstane-3,17-dione), 5β-androstanedione (5β-androstane-3,17-dione), 4-androstenedione (4-androstene-3,17-dione), norethynodrel (17α-ethynyl-17-hydroxy-5(10)-estren-3-one), norethindrone (19nor-4-androsten-17α-ethynyl-17β-ol-3-one), 19-nortestosterone (4-estren-17β-ol-3-one), β-sitosterol (24β-ethyl-5-cholesten-3β-ol), and estrone-3-sulfate (3-hydroxy-1,3,5(10)estratrien-17-one-3-sulfate) were obtained from Steraloids Inc. (Wilton, NH) except for dehydroepiandrosterone and moxestrol (RU 2858), which were obtained from Ikapharm (Ramat-Gan, Israel) and from Roussel Uclaf (Romainville, France), respectively.

The phytoestrogen coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Eastman Kodak (Rochester, NY) and genistein (4,5,7-trihydroxyisoflavone) and β-zearalanol (2,4-dihydroxy-6-[6β,10-dihydroxyundecyl]benzoic acid μ-lactone) were from Sigma (St. Louis, MO).

The synthetic estrogens diethylstilbestrol (4,4'-(1,2-diethyl-1, 2-ethenediyl)bisphenol), hexestrol (4,4'-(1,2-diethyl-1,2-ethane-diyl)bisphenol), and dienestrol (4,4'-(1,2-diethylidene-1,2-ethane-diyl)bisphenol) were obtained from Sigma. The antiestrogens tamoxifen (1-*p*-β-dimethylamino-ethoxyphenyl-*trans*-1,2-diphenylbut-1-ene), 4-OH-tamoxifen (1-(*p*-dimethylaminoethoxyphenyl)1-(4-hydroxyphenyl)-2-phenyl-but-1-ene), clomiphene (1-(*p*-(β-diethylaminoethoxy)phenyl)-1,2-diphenylchloro-ethylene), nafoxidine 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphtyl)-phenoxy]-ethyl)pyrrolidine hydrochloride, and ICI-164384 (*N*-*n*-butyl-11-(3,17β-dihydroxyestra-1,3,5(10)trien-7α-yl)-*N*-methylundecanamide) were obtained from Sigma or synthesized by KaroBio AB (ICI-164384) (Huddinge, Sweden). The environmental estrogens Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) and methoxychlor (1,1,1-trichloro-2,2-bis(*p*-methoxy-phenyl)ethane) were obtained from Aldrich (Germany). The structural formulas and chemical properties of all the competitors used can be found in the Merck Index or elsewhere (8–10).

Sephadex G25 columns (QS-2A) were obtained from Isolab (Akron, OH). All other chemicals were of the highest purity available.

In vitro transcription and translation

The 2.6 kbp rat ERβ cDNA (4) was subcloned into the *Eco*RI site of pBluescript (Stratagene, La Jolla, CA). The plasmid pT7βhER (11) containing the wild type (HEGO) human ERα sequence was a kind gift from Dr. B.W. O'Malley and co-workers (Baylor College of Medicine, Houston, TX). Human ERα and rat ERβ protein was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega, Madison, WI) with T7-RNA polymerase, during a 90 min reaction at 30 C. Translation reaction mixtures (50-μl portions) were snap-frozen and stored at −70 C until further use.

Saturation ligand binding analysis

Translation reaction mixtures were diluted in buffer A (20 mM HEPES, pH = 7.9; 150 mM NaCl, 10% wt/vol glycerol, 1 mM EDTA, 6 mM monothioglycerol, and 10 mM Na_2 MoO_4) and kept at 4 C. Aliquots equivalent to 0.25 μl ERα translation mixture or 2 μl ERβ translation mixture were incubated in duplo with 10–800 pM [^{125}I]-E_2 in the presence or absence of a 300-fold excess of diethylstilbestrol for 16 h at 4 C. The final incubation volume was 200 μl, and to the ERα incubation series unprogrammed reticulocyte lysate was added to equalize the total protein concentrations. Free and unbound radioligand was separated by gelfiltration over G-25 columns at 4 C as described (12). Bound radioactivity was measured in a Wallac γ-counter (Turku, Finland) with 70% efficiency. Specific binding was determined by subtracting nonspecific binding from total binding, and the free ligand concentration was estimated by subtracting total bound ligand from added ligand. The equilibrium K_d was calculated as the free concentration of radioligand at half-maximal binding by fitting data to the Hill equation (13) and by linear Scatchard transformation (14). Curve fitting was done in KaleidaGraph 2.1.3 (Abelbeck Software, PA).

Ligand competition experiments

Competitors were dissolved in dimethylsulfoxide at a concentration of 1 mM, except for coumestrol, genistein, and β-zearalanol, which were dissolved in ethanol. Translation reaction mixtures were diluted with

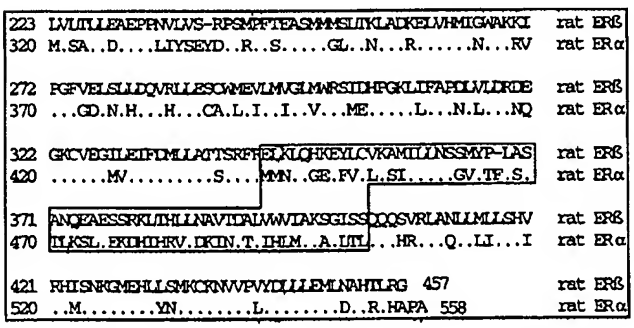

FIG. 1. Alignment of amino acid sequences of rat ERα protein (GenBank database Y00102) LBD (amino acid residues 320–558), and rat ERβ protein (GenBank database U57439) LBD (amino acid residues 223–457). For alignment, Clustal analysis using MEGALIGN/DNASTAR software was used.

buffer A and kept at 4 C. Aliquots equivalent to 0.25 μl ERα translation mixture or 2 μl ERβ translation mixture were added to dilutions containing [^{125}I]-E_2 and the respective competitors. The final concentration of radioligand was 125–150 pM, and the incubation time was 16 h at 4 C. Unprogrammed reticulocyte lysate was added to the ERα series to equalize protein concentrations. Competitors were present at concentrations between 10^{-4} M and 10^{-10} M; each competition curve consisting of eight concentrations in duplicates. Free and bound ligand were separated by gelfiltration over Sephadex G-25 columns as described (12). The data were evaluated by a nonlinear four-parameter logistic model (15) to estimate the IC_{50} value (the concentration of competitor at half-maximal specific binding). Relative binding affinity (RBA) of each competitor was calculated as the ratio of concentrations of E_2 and competitor required to reduce the specific radioligand binding by 50% (= ratio of IC_{50} values). The RBA value for E_2 was arbitrarily set at 100. The Cheng-Prusoff equation (13, 14) was used to calculate the K_i of the various competitors.

PCR analysis of rat tissue total RNA

Male and female rats (6–8 weeks old) were killed by cervical dislocation, and tissues were collected. Tissue samples were immediately processed for total RNA isolation according to the acid guanidinium thiocyanate-phenol-chloroform single-step extraction protocol (16). The integrity and quality of the purified RNA was controlled by formaldehyde denaturing agarose gel electrophoresis and by measurement of the A260/A280 nm ratio. Only RNA samples exhibiting an A260/A280 ratio >1.6 and showing integrity of the RNA by electrophoresis were used in further experiments. The RNA isolated from spleen and brain cortex appeared degraded and was discarded.

Random hexamer-primer cDNA synthesis was performed as described (17, 18). For the PCR amplification, 5% of the synthesized cDNA was added to a PCR reaction mixture as described (17) and amplified for 30 cycles by incubation at 95 C for 30 sec, 57 C for 15 sec, 72 C for 60 sec, and a final incubation at 72 C for 3 min, all in a PCR 9600 thermocycler (Perkin-Elmer, Norwalk, CT). The oligonucleotides erbkg1: 5′TTCCCGGCAGCACCAGTAACC (+38 relative to ATG) and erbkg2: 5′TCCCTCTTTGCGTTTGGACTA (+279 relative to ATG) were used for amplification of a 262-bp fragment of the ERβ messenger RNA (mRNA). The oligonucleotides kgb5: 5′AATTCTGACAATCGACGCCAG (+472 relative to ATG) and kgb6: 5′GTGCTTCAACATTCTCCCTCCTC (+794 relative to ATG) were used for amplification of a 344-bp fragment of the rat ERα mRNA. The oligonucleotides used for the amplification of actin mRNA are previously described (17). After agarose gel electrophoresis and blotting to nitrocellulose filters, the PCR products were hybridized to the internal oligonucleotides: ERUR 4: 5′GGGACTCTTTTGAGGTTCTGC (+163-182 relative to ATG) for ERβ, KG50: 5′GCAGCGAGAAGGGAAACATGA (+518-538 relative to ATG) for ERα, and actin primer: 5′GATGACCCAGATCATGTTTGA (+434-454 relative ATG) for actin according to a previously described protocol (17).

Results

Saturation ligand binding analysis of ER protein

The ER can be isolated from the cytosol of target cell extracts as a large nontransformed (*i.e.* non-DNA binding) 7–8S oligomeric complex, which contains hsp90 and hsp70 (2). It is believed that heat-shock proteins function to help fold the ER protein properly and to protect the hydrophobic hormone binding domain from inappropriate interactions (2). Rabbit reticulocyte lysates contain large amounts of several heat-shock proteins as hsp90 and hsp70, and have been used extensively for the study of ER complex formation with hsps, as well as for the study of requirements for steroid binding and interactions with DNA (2, 6, 11). When ERβ protein was synthesized *in vitro* and labeled with a saturating dose of [^{3}H]-E_2 and analyzed on sucrose density gradients, a single peak of specifically bound radioactivity was observed. The sedimentation coefficient of this complex was about 7S, and it shifted to 4S in the presence of 0.4 M NaCl (not shown). It was therefore decided to use human ERα and rat ERβ protein synthesized in reticulocyte lysates for the ligand binding experiments.

To obtain optimal conditions for the determination of equilibrium K_ds and RBAs of various ligands, the ER concentration in the binding assay was lowered to 10–20 pM. At these low ER concentrations radioligand and/or competitor depletion can be excluded while maintaining high receptor recovery during separation of bound and unbound ligand by the use of a gel filtration assay instead of the traditional charcoal adsorption assay (12). The low ER concentration made it necessary to employ radioiodinated estradiol as a probe, because the specific radioactivity of tritiated estradiol was too low to maintain sufficient accuracy. Radioiodinated E_2 (16α-[^{125}I]iodo-E_2) binds to the ER with high affinity and specificity as shown by its use in dry-mount autoradiographic techniques and various ligand binding assays (19, 20).

In Fig. 2 the result of a saturation ligand binding assay with [^{125}I]-E_2 is shown. Single point assays (not shown) were used to equalize the amount of ERα and ERβ protein used (10–15 pM). The nonspecific binding was ≤8% of total binding over the whole radioligand concentration range used. The K_d values calculated from the saturation curves (Fig. 2) were 0.06 nM for ERα protein and 0.24 nM for ERβ protein. Linear transformation of saturation data (Scatchard plots in Fig. 2) revealed a single population of binding sites for 16α-iodo-E_2 with a K_d of 0.1 nM for the ERα protein and 0.4 nM for the ERβ protein. The measured K_d values are in agreement with the finding that almost maximal stimulation of reporter gene activity by ERα and ERβ protein was previously found during incubation with 1 nM E_2 (4). Although the ERβ protein has a four times lower affinity for 16α-iodo-E_2 in this system compared with the ERα protein, both K_d values are within

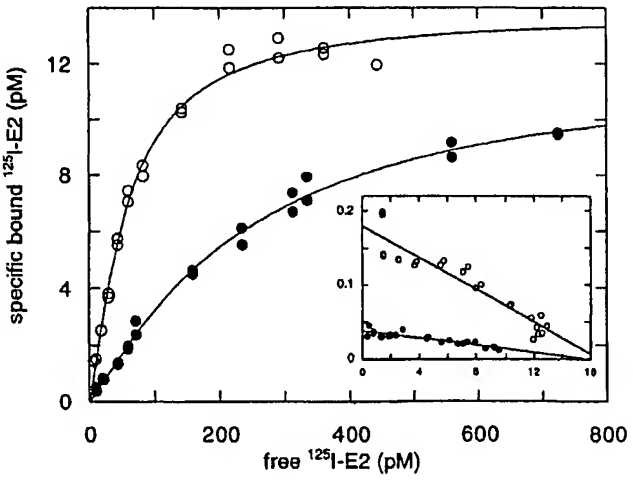

FIG. 2. Binding of 16α-[^{125}I]iodo-E_2 to *in vitro* synthesized ERα and ERβ protein in presence or absence of a 300-fold excess of diethylstilbestrol for 16 h at 4 C. Unbound radioactivity was removed as described, and specific bound counts (ERα = ○; ERβ = ●) were calculated by subtracting nonspecific bound counts from total bound counts. *Inset*, Scatchard analysis of specific binding giving a K_d of 0.1 nM for ERα protein and a K_d of 0.4 nM for ERβ protein.

the range (0.1–1 nM) generally reported for estradiol binding to ERs in various systems (1).

Ligand binding specificity of ERα and ERβ protein

Measurements of the equilibrium binding of the radioligand in the presence of different concentrations of unlabeled competitors provides readily interpretable information about the affinities of the latter, provided that radioligand and/or competitor depletion are avoided. Competition experiments were performed using ERα and ERβ protein concentrations of 10–15 pM and a [^{125}I]-E_2 concentration of about 150 pM, so that for both ERs the total receptor concentration was ≤0.1 K_d and the radioligand concentration was ≥10 times the ER concentration. Under such experimental conditions radioligand or competitor depletion can be excluded (14).

In total 37 substances were tested for both ER subtypes (Fig. 3 and Table 1). In Fig. 3 several examples of typical

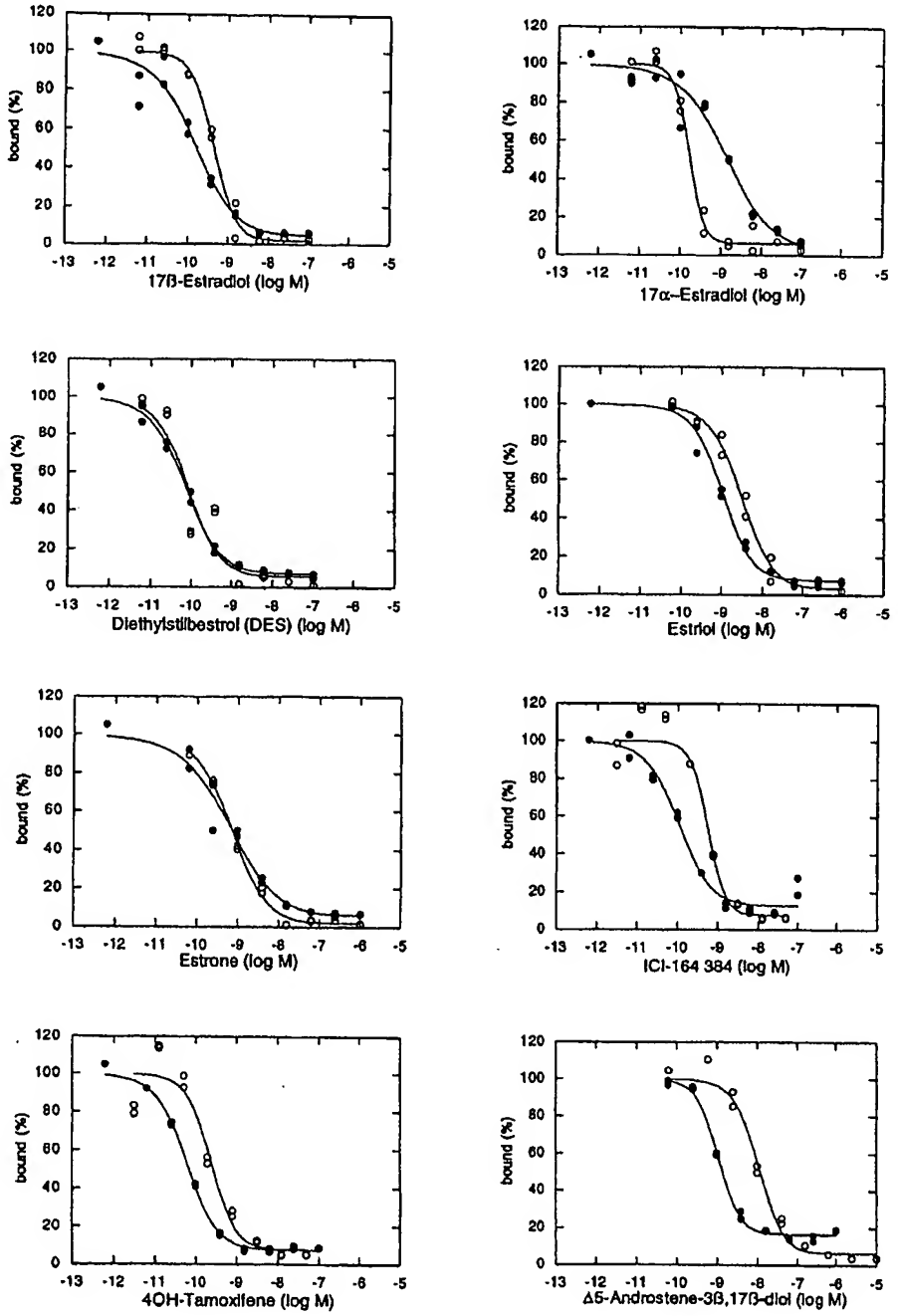

FIG. 3. Competition by several nonradioactive estrogenic substances and antiestrogens for 16α-[^{125}I]iodo-E_2 binding to *in vitro* synthesized ERα protein (○) and ERβ protein (●). Incubation was for 16 h at 4 C, and bound and unbound radioligand were separated as described.

TABLE 1. Binding affinity of various compounds for ERα and ERβ

Compound	RBA[a]		K_i (nM)[b]	
	ERα	ERβ	ERα	ERβ
E_2	100	100	0.13	0.12
Diethylstilbestrol	468	295	0.04	0.05
Hexestrol	302	234	0.06	0.06
Dienestrol	223	404	0.05	0.03
Estrone	60	37	0.3	0.4
17α-Estradiol	58	11	0.2	1.2
Moxestrol	43	5	0.5	2.6
Estriol	14	21	1.4	0.7
4-OH-Estradiol	13	7	1.0	1.9
2-OH-Estradiol	7	11	2.5	1.3
Estrone-3-sulfate	<1	<1		
4-OH-Tamoxifen	178	339	0.1	0.04
ICI-164384	85	166	0.2	0.08
Nafoxidine	44	16	0.3	0.8
Clomifene	25	12	0.9	1.2
Tamoxifen	7	6	3.4	2.5
5-Androstenediol	6	17	3.6	0.9
3β-Androstanediol	3	7	6	2
4-Androstenediol	0.5	0.6	23	19
3α-Androstanediol	0.07	0.3	260	48
5α-Dihydrotestosterone	0.05	0.17	221	73
Dehydroepiandrosterone	0.04	0.07	245	163
19-Nortestosterone	0.01	0.23	765	53
5α-Androstanedione	<0.01	<0.01		
Testosterone	<0.01	<0.01		
5β-Androstanedione	<0.01	<0.01		
4-Androstenedione	<0.01	<0.01		
Coumestrol	94	185	0.14	0.07
Genistein	5	36	2.6	0.3
β-Zearalanol	16	14	0.8	0.9
Bisphenol A	0.05	0.33	195	35
Methoxychlor	0.01	0.13	1774	90
Norethindrone	0.07	0.01	152	1084
Norethynodrel	0.7	0.22	14	53
Progesterone	<0.001	<0.001		
Corticosterone	<0.001	<0.001		
β-Sitosterol	<0.001	<0.001		

[a] RBA of each competitor was calculated as ratio of concentrations of E_2 and competitor required to reduce the specific radioligand binding by 50% (= ratio of IC_{50} values). RBA value for E_2 was arbitrarily set at 100. The IC_{50} of E_2 was 0.21 nM for ERα protein and 0.13 nM for ERβ protein.

[b] The Cheng-Prusoff formula (13, 14) was used to calculate the K_i of the various competitors.

competitor curves obtained are shown. In all cases monophasic curves were obtained for compounds with significant affinity. The slopes of the curves were almost similar, enabling the use of IC_{50} values to calculate RBA values (Table 1), with an RBA value of 100 for E_2 for each receptor. For the ERα as well as ERβ protein, the estradiol binding was stereospecific because 17α-estradiol showed a two times and 10 times lower affinity, respectively (Table 1), compared with E_2, which is in agreement with previous findings on stereospecific binding of estradiol by the ER (21). However, in making such comparisons, it should be kept in mind that most, if not all, ER ligand binding studies done in the past 30 yr actually involved mixtures of ERα and ERβ protein. This is certainly the case for many studies in which rat uterus cytosol was used (see following). The present study is the first in which the ligand binding properties of both ER subtypes are measured separately, and caution is needed when comparing RBAs from this study with the previous studies involving mixtures of ER subtypes.

For the physiological estrogens, the order of competition was E_2 > estrone, 17α-estradiol (ERα) > estriol > catecholestrogens, 17α-estradiol (ERβ) > estrone-3-sulfate. Overall the ERα and ERβ proteins show the binding characteristics and relative affinity for the physiological estrogens found to be characteristic for an ER protein (1, 8, 22). The stilbene estrogens, which consist of a composite diphenolic ring structure, bind with high affinity to both ER subtypes. However, different orders of competition were found: diethylstilbestrol > hexestrol > dienestrol > (E_2) for ERα and dienestrol > diethylstilbestrol > hexestrol > (E_2) for ERβ.

The extra methoxy group at C11 and the ethynyl group at C17 of moxestrol (RU 2858) lowered the affinity compared with E_2 for the ERα protein by only a factor of 2 but for the ERβ protein by a factor of 20. Moxestrol is in use as a radioligand in ER assays, and is known to have a lower binding affinity than E_2 under certain assay conditions (23).

The triphenylethylene (anti)estrogens were developed by successive chemical modifications of the triphenylethylene nucleus, formed by the addition of an extra phenyl ring to the stilbene nucleus as present in for instance diethylstilbestrol (9, 10). Interestingly, the measured order of affinity for the tested triphenylethylene (anti)estrogens was the same for both ER subtypes: 4OH-tamoxifen ≫ nafoxidine > clomifene > tamoxifen. The steroidal antiestrogen ICI 164384 had a high affinity for ERα as well as for ERβ, confirming that extensions at C7 do not preclude ligand-ER interactions (9, 24).

It has been known for a long time that a number of compounds classified as androgens (C19 steroids) can evoke estrogen-like effects in the female genital tract and in the mammary glands (25). Of all the androgens tested only those with a hydroxyl group at C3 and C17 had significant affinity for both ER subtypes (Table 1). The relative flatness of the A-ring with respect to the B-ring is also important, given the clear difference in affinity for both ER subtypes between 5-androstenediol and 4-androstenediol. The binding affinity of 3β-androstanediol and 5-androstenediol for both ER subtypes is in agreement with previous studies showing specific binding to the rat uterus ER and estrogenic responses in rat uterus and mammary tumors for both steroids (26, 27).

Norethynodrel and norethindrone, progestins derived from 19-nor-testosterone, and 19-nor-testosterone itself have an intrinsic estrogenic potential as shown by the induction of alkaline phosphatase activity in ER-positive human endometrial cancer Ishikawa cells (28). The apparent binding affinity of norethynodrel and norethindrone for both ER subtypes was however, only about 1/500th of that for E_2 (Table 1), and the need for a conversion into more active metabolites by aromatization or hydroxylation at C-3 has been suggested (28).

Several plant-derived nonsteroidal compounds such as genistein and coumestrol have estrogenic activity (8). These compounds increase rat uterine weight and stimulate growth of breast tumor cells and compete with E_2 for binding to ER protein as well as stimulate the activity of reporter genes in the presence of ER protein (Ref. 29 and references therein). Both coumestrol and genistein had a significantly higher

affinity for ERβ protein (Table 1), which is interesting in the light of the high expression of ERβ mRNA in the secretory epithelial cells of the prostate, and the prostate cancer protective properties that have been associated with these compounds (30). Zearalanols are fungal metabolites or derivatives thereof that have been associated with estrogenizing syndromes in cattle fed with mold-infected grain (8). Despite the fact that zearalanols are structurally very different to known steroidal and nonsteroidal estrogens, they interact with the rat uterus cytosolic ER (31). Also, in our competition assays β-zearalanol interacted with both ER subtypes with a similar affinity (Table 1), as was reported previously for the rat uterus ER protein (31).

Abnormal sexual development in reptiles as well as the increasing incidence of certain human reproductive tract abnormalities (such as hypospadias) has been associated with increased exposure to and body burdens of so-called estrogenic environmental chemicals (32, 33). These effects from estrogenic chemicals as, for instance, the pesticide methoxychlor and the plastics ingredient bisphenol A, are postulated to be mediated via the ER because these compounds have estrogenic effects (increase of uterine weight) in female rats (8, 32, 33). Bisphenol A and methoxychlor both inhibited the binding of [^{125}I]-E_2 by the ERα and ERβ protein, and the inhibition seemed to be stronger for the ERβ protein (Table 1). However, it was clearly a very low affinity interaction, and the fact cannot be excluded that it involved different sites on the ER than those involved in the binding of E_2.

Expression of ERα and ERβ mRNA in rat tissues

To determine the relative distribution of ERα and ERβ mRNA, total RNA was isolated from rat tissues and used for RT-PCR using primers specific for each ER subtype. All tissues were taken from 6- to 8-week-old male rats, except uterus and ovary, which were taken from 8-week-old female rats. Although this assay was only semiquantitative, it is clear that the relative distribution of both ER subtypes was quite different (Fig. 4). Highest expression of ERβ mRNA was found in the ovary and prostate, which is in agreement with our previous *in situ* hybridization experiments using male and female rats of similar ages (4). In addition, testis, uterus, bladder, and lung showed moderate expression, whereas pituitary, epididymis, thymus, various brain sections, and spinal cord reveal low expression of ERβ mRNA. The ERα mRNA was highly expressed in epididymis, testis, pituitary, uterus, kidney, and adrenal, which all showed moderate or no expression of ERβ mRNA. Aside from weak expression in thalamus/hypothalamus, the brain sections tested were negative for ERα mRNA. Ovary and uterus, which are known to contain high amounts of ER protein (1), clearly expressed both ER subtypes. All organs from male rats previously described to display specific binding of [^{3}H]-E_2 to an 8S cytosolic protein, *i.e.* liver, lung, adrenal, pituitary, prostate, epididymis, and testis, showed clear expression of either ER subtype mRNA or both (34, 35).

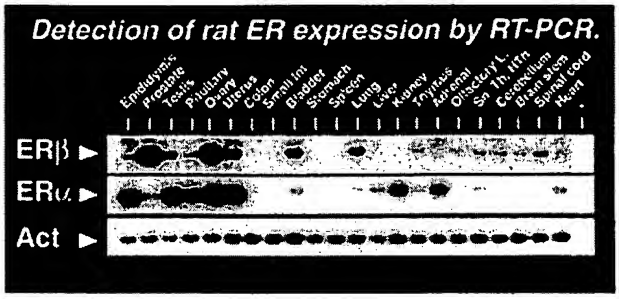

FIG. 4. Rat tissue distribution of ERα mRNA and ERβ mRNA determined by RT-PCR (see *Materials and Methods*). Autoradiograms are shown of blots after hybridization with oligonucleotide probes specific for ERβ (*top*), ERα (*middle*), and actin (*bottom*). Sn, Substantia nigra, preopticus; Th, thalamus; Hth, hypothalamus; Olfactory L., olfactory lobes; Small Int., small intestine.

Discussion

The ligand binding affinity of ERα and ERβ protein is overall quite similar for the physiological ligands, certainly when only the order of competition is compared. The most interesting difference was found for 17α-estradiol, which has a five times higher affinity for ERα protein. The physiological action of 17α-estradiol is quite different from that of E_2, because 17α-estradiol is a short-acting estrogen and actually a time-dependent mixed agonist-antagonist in the rat/mouse uterus (1). Short-acting estrogens (estrone, estriol, and 17α-estradiol) do cause nuclear binding of the hormone-receptor complex but only for a short period of time (1). It would be interesting to see whether the difference found for 17α-estradiol in our ligand binding assays is also present in a transactivation assay system. For the other types of ligands tested, *i.e.* antiestrogens, androgenic steroids, and phytoestrogens, there are some interesting differences in the RBAs (Table 1), but it remains to be seen whether these differences are also reflected in a transactivation assay system using different cellular backgrounds. The ERβ protein clearly displays all the ligand binding characteristics of a classical ER protein (1, 8–10, 21, 22, 26–29, 31, 36), and therefore it seems unlikely that a unique physiological ligand for the ERβ protein exists. The relative order of ligand binding for various estrogens (diethylstilbestrol, estrone, and estriol) is slightly different in this study than in our previous study (4). Our previous rather preliminary assay (4) was hampered by relatively high levels of nonspecific radioligand binding (30–40% compared with about 5% in this report), which might explain the difference.

A question of considerable interest is why, despite the numerous ligand binding assays performed for the ER protein, an indication for the existence of two ER subtypes was never published. Of course, distinguishing between a mixed population of receptor subtypes and a homogeneous receptor population by saturation or homologous/heterologous competition assays is generally difficult. This is only possible with certainty when the two subtypes differ sufficiently in affinity (10- to 100-fold), and the range of ligand concentrations examined is wide. Furthermore, the proportions of the two subtypes must be appropriate (37). Of all the radioligands used in ER assays (E_2, DES, hexestrol, moxestrol, 16α-iodo-estradiol), the difference in affinity for moxestrol between both ER subtypes is the greatest (8-fold) in our experiments. In this regard, it should be realized that to detect the existence of receptor subtypes the higher affinity

subtype should be less abundant than the lower affinity subtype (37). Most ER ligand binding assays have been done with uterus extracts and breast tumor extracts or cell lines, and it could be that the right conditions for the detection of receptor subtypes are not fulfilled in these cases. We have been unable to detect the ERβ mRNA in various breast tumor cell lines (MCF-7, T47D, ZR75-1) by RT-PCR (our unpublished observations), whereas both subtypes are expressed in rat (Fig. 4) and human uterus (not shown).

In prostate and ovary, the two tissues that express high levels of ERβ mRNA, it has been difficult to demonstrate the presence of ERs by immunostaining with the available ER antibodies, although specific binding of E_2 could be measured (1, 8, 34). In human and rat prostate at best only weak staining in stromal smooth muscle cells was found (38, 39), which is in contrast with our results showing high expression of ERβ mRNA in the prostate secretory epithelial cells (4). In the rat and human ovary, specific binding of estradiol was found in intact follicles and granulosa cells (40–41), but no ER could be detected with available ER antibodies (41). These discrepancies could be explained by the fact that the most frequently used ER protein antibodies, H-222 and H-226 (42), do not cross-react with rat ERβ protein on immunoblots (our unpublished observations). The above findings and our results could indicate that the ERβ protein is the predominant if not the only ER subtype present in rat prostate and ovary. Of course this remains to be proven when specific ERβ protein antibodies become available. The fact that disruption of the ERα gene *in vivo* did not eliminate the ability of small follicles to grow, as is evident from the presence of secondary follicles and antral follicles in the ERα knockout mouse (43), also argues for the presence of alternative ER (ERβ?) molecules. In fact, rat uterus, ovary, testis, epididymis, and pituitary clearly express both ER subtypes mRNAs. Although we have no data on ERβ mRNA expression or protein concentration in tissues of the ERα knock-out mouse (43), the possible presence of ERβ protein should be kept in mind when interpreting experiments using the ERα knock-out mouse. Furthermore, in the uterus of the ERα knock-out mouse, residual E_2 binding could be measured (43), which is likely caused by the presence of ERβ protein. In the brain of the ERα knock-out mouse, specific binding of E_2 and modulation of progesterone receptor gene expression by E_2 was observed (44). Again this is most likely caused by the presence of ERβ protein, because the ERβ mRNA is broadly expressed in the rat brain and probably also in the mouse brain at a low level. Detailed mapping of ERα and ERβ expression in rat/mouse brain by *in situ* hybridization or using specific antibodies for each subtype is of clear interest given the fact that for the localization of ERs in the brain of various species antibodies that do not recognize ERβ have been used (45).

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Clinical Pharmacokinetics of Endocrine Agents Used in Advanced Breast Cancer

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Summary Endocrine therapy is important in the treatment of advanced breast cancer. The prototype antiestrogen tamoxifen and the prototype aromatase inhibitor aminoglutethimide have been in clinical use for more than 2 decades, as have synthetic progestin derivatives. Currently, several novel antiestrogens and aromatase inhibitors are used to treat breast cancer. This paper reviews the present knowledge of the clinical pharmacokinetics of these drugs. Drug monitoring in plasma and other body fluids has been improved over recent years by the introduction of sensitive and specific high performance liquid chromatography and gas chromatography-mass spectrometry methods. However, we still lack information on such basic pharmacokinetic parameters as the bioavailability of several of these drugs. It is important to study not only plasma but also tissue drug concentrations.

Breast cancer is the most frequent malignancy among women in Western countries. Despite improved prognosis due to earlier diagnosis, 40 to 50% of patients will develop metastatic disease (Rutqvist 1984) for which no cure is available. However, with proper treatment many patients achieve remission and good palliation. The 2 major goals of systemic treatment are to prevent progression to metastatic disease and to cause remission and optimal palliation in patients with metastatic disease. The 2 major systemic treatment modalities available to treat patients with breast cancer are endocrine therapy and chemotherapy.

In patients with estrogen receptor positive tumours, the chances of a response to hormone therapy may be as good as those for a response to aggressive chemotherapy (Degenshein et al. 1980; Henderson 1983; Gundersen & Kvinnsland 1986; McGuire 1978; Vollenweider-Zerargui et al. 1986). Hormone therapy causes fewer side effects and is better tolerated. Endocrine treatment is thus the first line of therapy for patients with tumours expressing the estrogen receptor or for patients with long disease-free intervals which suggest a hormone-sensitive tumour.

Several trials confirm that adjuvant therapy with tamoxifen reduces the risks of relapse and death [Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 1988]. Patients responding to 1 hormone therapy may, after relapse, subsequently respond to other forms of hormonal treatment. The main goal of contemporary endocrine treatment for breast cancer is to suppress estrogen stimulation of the breast cancer cell, either by suppressing estrogen hormone levels or by blocking estrogen stimulation with antiestrogens.

This paper reviews our current knowledge of the disposition of endocrine drugs used to treat breast cancer. Four major classes of drugs are discussed: GnRH agonists, antiestrogens, aromatase inhibitors and high dose progestins.

1. Gonadotrophin Releasing Hormone Analogues

Since the discovery of the structure of the decapeptide gonadotrophin releasing hormone (GnRH, luteinising hormone releasing hormone, LHRH) [Schally et al. 1971], a new class of pharmacological agents has emerged, the GnRH analogues. These analogues have a similar chemical structure to GnRH but are substituted at position 6 with or without modifications in the C-terminal sequence. GnRH is found in the hypothalamus of mammals. It is released in pulses into the hypothalamo-hypophyseal vein (Knobil 1980) and interacts with specific (possibly adenylate cyclase linked) receptors on the adenohypophyseal cells (Clayton & Catt 1981). This stimulates gonadotrophin release, which controls the secretion of estrogens and androgens from the gonads.

GnRH analogues are in clinical use in such diverse areas as infertility (Donnelly 1987), benign gynaecological conditions (Brogden et al. 1990;

Donnelly 1987) and oncology. On the basis of results with GnRH analogues in animal breast tumour models (Nicholson & Maynard 1979), the first results from use of a GnRH analogue (buserelin) in premenopausal women with breast cancer appeared in 1982 (Klijn & DeJong 1982).

Since 1971, more than 1000 different modifications of GnRH have been made in an effort to produce more powerful analogues. Modifications are guided by knowledge of the sites of enzymatic attack of the decapeptide. All analogues in clinical use are either decapeptides or nonapeptides with modification in position 6, where the amino acid L-glycine has been exchanged with a D-amino acid [triptorelin, D-Trp^6 GnRH; nafarelin, (D-Nal$_2$)^6 GnRH] or where in addition the C-terminal amino acid is replaced with either ethylamide [buserelin, D-Ser(Bu^t)^6 Pro^9-ethylamide-GnRH] or substituted for a modified amino acid [goserelin, D-Ser(Bu^t)^6 Aza-Gly^{10} GnRH]. The main effects of these modifications are increased affinity for the GnRH receptor and decreased susceptibility to enzymatic degradation (Nestor 1984; Robinson & Jordan 1988; Sandow 1987).

1.1 Biochemical Actions

GnRH analogues were developed to stimulate gonadotrophin release, but it was soon realised that while short term use greatly stimulated gonadotrophin release, prolonged administration led to profound inhibition of the pituitary-gonadal axis (Nillius 1981). Plasma estradiol and progesterone are consistently suppressed to postmenopausal or castrate levels after 2 to 4 weeks of treatment with buserelin, goserelin or leuprorelin (leuprolide) [Harvey et al. 1987; Klijn et al. 1984; Nicholson & Walker 1989]. Minor plasma estradiol suppression in postmenopausal women may be due to inhibited ovarian testosterone secretion (Dowsett et al. 1988).

Alternative mechanisms of action of GnRH analogues in breast cancer are controversial. High affinity GnRH receptors are found in several normal tissues including the mammary gland (Clayton et al. 1979; Hsueh & Jones 1981; Lefebvre et al. 1980; Liscowitch & Koch 1982) and in breast cancer (Butzow et al. 1987; Ciocca et al. 1990; Eidne et al. 1985; Fekete et al. 1989). GnRH analogues inhibit breast cancer cell growth *in vitro*, but the role of direct mechanisms of action of GnRH analogues *in vivo* is still in doubt.

1.2 Clinical Experience

Since Klijn and DeJong (1982) described an objective response in 2 of 4 women with breast cancer treated with buserelin, several reports of the effect of GnRH analogues on premenopausal breast cancer have appeared (Harvey et al. 1987; Høffken et al. 1988; Klijn et al. 1985; Robertson et al. 1989). A summary of the accumulated published data shows an objective response in 45 of 123 assessable patients; a response rate of 37% (Sheth & Allegra 1991). Results from phase II trials of goserelin show an objective response in 36% of 228 patients (Blamey R, personal communication). These results compare favourably with those after ovarian ablation in a similar group of patients (Henderson 1987). GnRH analogues in postmenopausal breast cancer (Harris et al. 1989; Nicholson et al. 1989; Plowman et al. 1986) have an overall response rate of about 10%. Whether this is due to a slight suppression of plasma estradiol (Dowsett et al. 1988) or other mechanisms of action remains unclear.

1.3 Clinical Pharmacokinetics

The pharmacology and pharmacokinetics of individual GnRH analogues have recently been reviewed meticulously (Brogden et al. 1990; Chrisp & Goa 1990, 1991). Only the findings of greatest relevance to breast cancer treatment are given here.

1.3.1 Drug Measurement

Buserelin and goserelin are measured in plasma and urine by radioimmunoassay (RIA) with and without high performance liquid chromatography (HPLC) separation from the metabolites (Clayton et al. 1985; Holland et al. 1986; Kiesel et al. 1989; Perres et al. 1986).

1.3.2 Absorption

Generally, polypeptides are not administered orally. They are poorly absorbed due to their high molecular weight and undergo enzymatic degradation in the digestive tract. Intranasal, intravaginal and percutaneous administration show varying and unpredictable absorption, whereas subcutaneous, intramuscular or intravenous administration assure complete absorption (see Brogden et al. 1990; Chrisp & Goa 1990; Hutchinson et al. 1987; Robinson & Jordan 1988 for references to the original works). Due to short elimination times, clinical efficacy requires frequent administration. Parenteral sustained release microcapsule preparations exist (Chien 1981), but have only recently come into practical use, as for the GnRH analogue leuprorelin (Crighton et al. 1989; Dowsett et al. 1990a). GnRH analogues are microencapsulated in polylactic and polyglycolic acid and suspended in saline. Homo- and copolymers of lactic and glycolic acids are inert and biocompatible, degrading to toxicologically acceptable products. This material was chosen to make a polymer rod for subcutaneous injections from which goserelin is continuously released (Hutchison & Furr 1990). Single, subcutaneous depots of goserelin or buserelin result in controlled drug release for several weeks (Ahmann et al. 1986; Blom et al. 1989), a convenient and safe method of administration. Recently, possible delivery of GnRH analogues by inhaled aerosol has been suggested (Adjei & Garren 1990).

1.3.3 Metabolism and Excretion

GnRH analogues are metabolised in the liver, kidney, hypothalamus and pituitary gland (Bennett & McMartin 1979; Parker et al. 1979; Robinson & Jordan 1988). The main metabolic pathways are enzymatic cleavage of the peptide bond between Tyr^5 and the amino acid in position 6 by neutral peptidases and cleavage of the peptide bond between Pro^9 and $Gly\text{-}NH_2{}^{10}$ by the postproline cleaving enzyme (see Robinson & Jordan 1988 for references). In general, substitution on Gly^6 and modifications in the C-terminal $Gly\text{-}NH_2$ seem to make the analogues more resistant to enzymatic degradation.

Studies of buserelin indicate some biological activity of its metabolites in animals (Sandow et al. 1980), but the relevance of this to the use of GnRH analogues in human breast cancer is unknown.

1.3.4 Plasma and Tissue Pharmacokinetics and Drug Interactions

GnRH is rapidly removed from the plasma after intravenous injection in humans. Its plasma pharmacokinetics best fit a 2-compartment model with an initial half-life ($t_{1/2}$) of about 2 to 12 min and a terminal $t_{1/2}$ of 30 to 60 min (Bennett & McMartin 1979). Similar pharmacokinetic patterns are found for GnRH analogues. Some, like triptorelin and nafarelin, have a longer terminal $t_{1/2}$ of about 120 min (Barron et al. 1982; Chan et al. 1988). The mean terminal $t_{1/2}$ was about 5h after subcutaneous goserelin in male patients with prostatic cancer (Clayton et al. 1985). These investigators reported a total body clearance (CL) of 1.6 to 2.5 L/h (mean value of 1.9 L/h) in 6 patients receiving subcutaneous infusion. Adam et al. (1988) found a higher CL (about 8 L/h). Clayton et al. (1985) reported an apparent volume of distribution (Vd) of goserelin in humans of about 14L, corresponding to extracellular body water. Thus, their results showed good consistency between CL and steady-state plasma concentrations (C^{ss}) of the drug.

Except for nafarelin, which has a plasma protein binding of about 80% (Chan & Chaplin 1985), GnRH analogues are only slightly bound to plasma proteins (Chrisp & Goa 1991; Clayton et al. 1985; Sandow et al. 1987). When radioactive tracers of goserelin or buserelin are given to animals, high concentrations of radioactivity are found in the pituitary, kidneys and liver (Chrisp & Goa 1991; Sandow & Konig 1979). The low Vd for goserelin suggests this drug may not concentrate in any major tissue compartment.

GnRH analogues are not known to interact with the pharmacokinetics of other drugs.

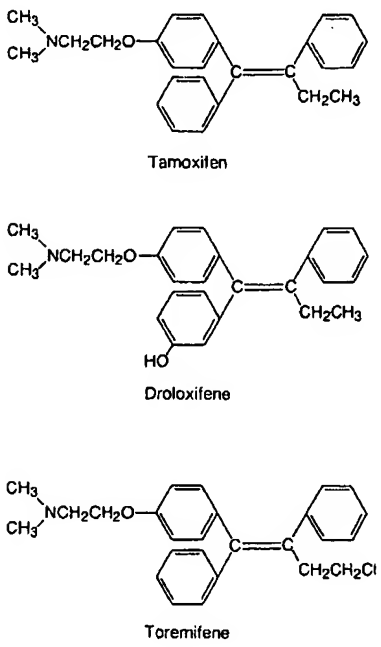


Fig. 1. Chemical structure of antiestrogens currently used in breast cancer treatment.

2. *Antiestrogens*

The ability of nonsteroidal compounds to block estrogen action *in vivo* was first documented with ethamoxytriphetol (MER-25) [Barany et al. 1955; Lerner et al. 1958]. Since then, several compounds to treat breast cancer were investigated, but studies were discontinued due to adverse CNS effects (Holtkamp et al. 1960; Kistner & Smith 1960). Subsequent trials tested several compounds until tamoxifen proved to be an effective endocrine therapy with few side effects (Legha & Carter 1976).

2.1 Biochemical Actions of Tamoxifen

The structure of tamoxifen is shown in figure 1. The drug is thought to act by blocking estrogen stimulation of breast cancer growth, mimicking the first step of estrogen action, the promotion of both translocation and nuclear binding of the estrogen receptor (Borgna & Rochefort 1981; Jordan et al. 1977). The basis of the antagonism may be that tamoxifen alters transcriptional and post-transcriptional events (Lau et al. 1991; Webster et al. 1988).

The action of tamoxifen *in vivo* may be more complex. Certain metabolites have important biological activities. It is thought that 4-hydroxytamoxifen (metabolite B) may be partly responsible for the effects of tamoxifen *in vivo* (Allen et al. 1980; Borgna & Rochefort 1981). Tamoxifen can behave either as a frank estrogen (pure agonist), a partial agonist or as an antagonist, depending on the species, target organs and end-points assessed (Furr & Jordan 1984; Jordan 1984; Jordan et al. 1990).

Alternative mechanisms of action of tamoxifen have been suggested. The drug and its metabolites may inhibit the conversion of estrone sulfate to estradiol (Gelly & Pasqualini 1988; Pasqualini & Gelly 1988), bind to 'antiestrogen binding sites' (AEBS) [Brandes & Bogdanovic 1986; Sutherland et al. 1980] and inhibit protein kinase C (Horgan et al. 1986; O'Brian et al. 1985) or calmodulin (Lopes et al. 1990; Rowlands et al. 1990).

2.2 Clinical Experience with Tamoxifen

Tamoxifen is the most extensively used endocrine treatment for breast cancer. As first-line treatment to postmenopausal women with advanced breast cancer it may cause objective remission (Hayward et al. 1977) in 30 to 40% of unselected patients (Jordan et al. 1988). The response rate is 50 to 70% in patients expressing a positive estrogen receptor value (McGuire 1978; Vollenweider-Zerargul et al. 1986). Objective remissions also occur in premenopausal breast cancer (Santen et al. 1990). Successful tamoxifen use as adjuvant therapy of breast cancer (EBCTCG 1988) is among the most impressive improvements in treatment of the disease during the last decades. The beneficial profile of the drug has suggested its use in the prevention of breast cancer (Fentiman 1989, 1990; Gazet 1985; Powles et al. 1990).

Tamoxifen causes few adverse effects (Patterson et al. 1982). Long term use may be associated with

a modest increase in the risk of development of endometrial carcinoma (Fornander & Rutqvist 1989). Other side effects may be beneficial. The drug seems to maintain bone density (Spicer et al. 1991; Wolter et al. 1988) and reduce serum cholesterol (Love et al. 1990; Powles et al. 1990; Rossner & Wallgren 1984), LDL cholesterol (Caleffi et al. 1988; Love et al. 1990; Powles et al. 1990; Rossner & Wallgren 1984) and apolipoprotein B (Powles et al. 1990) whereas a small decrease in HDL cholesterol and increase in triglyceride levels take place (Love et al. 1990). These data suggest a positive effect of tamoxifen on lipoprotein metabolism and may explain why the drug seems to have a cardioprotective effect in postmenopausal women when administered for more than 5 years (McDonald & Stewart 1991).

2.3 Clinical Pharmacokinetics of Tamoxifen

2.3.1 Drug Measurement

Plasma concentrations of tamoxifen and its metabolites are below the concentrations detected by HPLC analyses using regular UV-detection. Adam et al. (1980a) found that tamoxifen and many of its metabolites may be converted into highly fluorescent products by UV-irradiation, making it possible to measure these compounds in plasma from patients receiving tamoxifen therapy (table I, fig. 2).

2.3.2 Absorption

Surprisingly, the absorption of tamoxifen has not been properly evaluated. Comparison of the plasma area under the concentration-time curve (AUC) following oral and parenteral administration has not been performed although indirect evidence suggests that th ˙¬ug is well absorbed. Rats administered intraperitoneal [^{14}C]tamoxifen and dogs given oral [^{14}C]tamoxifen showed a similar excretory pattern of radioactivity in the faeces and urine (Fromson et al. 1973a).

When oral [^{14}C]tamoxifen was administered to 2 women with breast cancer, 26 and 51% of the radioactivity was slowly excreted in the faeces and as little as 9 and 14% appeared in the urine over 8 and 12 days, respectively (Fromson et al. 1973b). Bioavailability from tablets and aqueous solution was similar (Adam et al. 1980), but the relative bioavailability was only 30% when the drug was administered in suppositories rather than orally (Tukker et al. 1986).

Good tamoxifen absorption does not exclude possible significant first-pass metabolism. Fromson et al. (1973b) administered radioactive tamoxifen orally to 2 women, and unchanged drug accounted for only about 30% of total plasma radioactivity 1 to 4h after ingestion. Thus, the bioavailability of tamoxifen is not known.

2.3.3 Metabolism and Excretion

The metabolism of tamoxifen is complex (fig. 2). Several metabolites are thought to be biologically active and some may be more potent than tamoxifen itself (Adam et al. 1980; Robertson et al. 1982). Thus, some of the metabolic pathways may not result in inactivation but rather activation of the drug.

When [^{14}C]tamoxifen was given orally to rats, mice, dogs and rhesus monkeys, 58 to 105% of the radioactivity was detected in the faeces and 2 to 21% in the urine over a period of 10 to 20 days after drug intake (Fromson et al. 1973). Most of the labelled material in both urine and faeces was excreted as glucuronides, the rest as other conjugates and unidentified polar metabolites.

In patients given [^{14}C]tamoxifen, approximately 50 to 70% of the radioactivity in faecal extracts represented polar conjugates and 5 to 8% was found as metabolite F (Fromson et al. 1973). Bile and urine contain conjugates of the hydroxylated metabolites Y, B, and BX, whereas in faeces metabolite B and tamoxifen predominate (Lien et al. 1989).

Substantial amounts of radioactivity excreted into the bile of rats and dogs are later reabsorbed and undergo enterohepatic circulation. Little appears in the urine. Enterohepatic circulation may contribute to the long $t_{1/2}$ and extended biological activity of tamoxifen (Fromson et al. 1973).

The hydroxylated metabolites, once formed, may be rapidly conjugated and eliminated via the bile, which could explain the low or undetectable con-

Table I. Methods for evaluation of tamoxifen and its metabolites in plasma/serum

Method	Compound	DL (μg/L)	Feature	Advantage	Disadvantage	Reference
HPLC	T,B	1-2	UVC before separation, no IS	Sensitive	Evaporation	Mendenhall et al. (1978)
GC-MS	T	<10	IS	High specificity	Evaporation	Gaskell et al. (1978)
GC-MS	T, B	1	IS	Sensitive, high specificity	Evaporation, 2 runs	Daniel et al. (1979)
TLC	T, X	2.5	UVC after separation, IS	Inexpensive equipment	Evaporation	Adam et al. (1979, 1980a,b)
HPLC	T, B, X	0.1	No IS, pre-column UVC	High sensitivity	Evaporation	Golander & Sternson (1980)
GC-MS	T, B, X	1	IS	High specificity	Laborious	Daniel et al. (1981)
TLC	T, X, Y, Z	ND	UVC after separation, IS	Multiple metabolites detected	Evaporation, B not detected, not baseline	Kemp et al. (1983)
HPLC	T, B, Y, X	2	Post-column on-line UVC, IS	Multiple metabolites detected	Evaporation, 3 runs	Brown et al. (1983)
HPLC	T, B, X	<1	Post-column on-line UVC, IS	High sensitivity	Sep-Pac extraction, vacuum concentration	Camaggi (1983)
HPLC	T, B, X	8	UVC before separation, IS		Low sensitivity, evaporation	Wilbur et al. (1985)
HPLC	T, B, X, Y, Z	1-2	Post-column on-line UVC, IS	Good baseline separation		Milano et al. (1987)
HPLC	T, X	<0.1	Post-column on-line UVC, no IS	High sensitivity	Evaporation	Nieder & Jaeger (1987)
GC-MS	T, B, X, Y	0.05-0.3	IS	Precise, accurate, specific, protects labile catechol metabolites	Multiple steps	Murphy et al. (1987)
HPLC	T, B, BX, X, Y, Z	<1	Post-column on-line UVC, IS	Automated, high output, guard column	Y interferes with solvent front from tissue samples	Lien et al. (1987, 1988)
HPLC	T, B, E, X, Y, Z	5.0	Pre-column UVC, IS	Multiple metabolites detected	Not baseline separation, evaporation, separate run for X	Stevenson et al. (1988)
HPLC	T, Y, B, X, E	0.6	Post-column on-line UVC, IS	Automated, multiple metabolites detected	2 runs, evaporation and extraction	Langan-Fahey et al. (1990)
	BX	ND				
HPLC	T	6	Direct injection, internal surface packing	Automated	Complicated, only T detected	Matlin et al. (1990)

Abbreviations: IS = internal standard; UVC = ultraviolet conversion; HPLC = high performance liquid chromatography; GC-MS = gas chromatography-mass spectrometry; TLC = thin layer chromatography; DL = detection limit in plasma or serum; ND = not determined; T = tamoxifen; B, BX, E, X, Y, Z = tamoxifen metabolites.

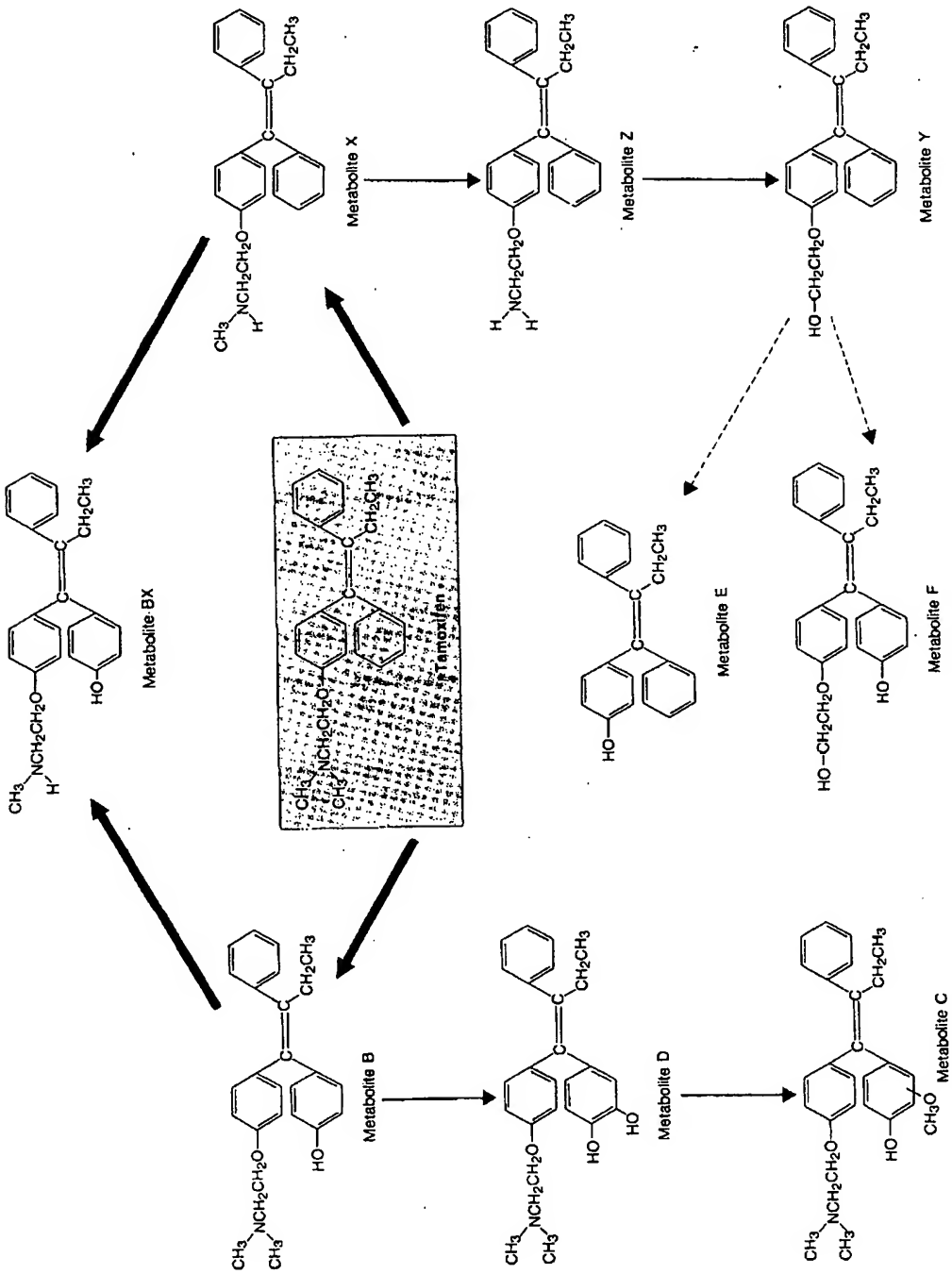


Fig. 2. Proposed metabolic pathways for tamoxifen in humans.

centrations of these metabolites in serum (Adam et al. 1979).

Of tamoxifen metabolites (fig. 2), metabolite Y, the primary alcohol of tamoxifen, has a glycol side-chain and is a minor metabolite with low binding affinity for the estrogen receptor and weak anti-estrogenic activity (Jordan et al. 1983; Kemp et al. 1983). It has been detected in plasma, urine and faeces (Brown et al. 1983; Jordan et al. 1983; Lien et al. 1989).

Metabolite B (4-hydroxy-tamoxifen) is a minor metabolite with high affinity for the estrogen receptor and high antiestrogenic potency (Jordan et al. 1977a). It may be responsible for a major part of the effects of tamoxifen *in vivo* and is present in plasma, urine, faeces and all tissues examined (Daniel et al. 1979, 1981; Fabian et al. 1981; Lien et al. 1991a).

Metabolite BX (4-hydroxy-*N*-demethyl-tamoxifen) is formed after percutaneous administration of metabolite B to humans (Mauvais-Jarvis et al. 1986) and is detected in the bile and plasma of patients receiving oral treatment (Langan-Fahey et al. 1990; Lien et al. 1988). Despite a low plasma concentration, BX may be of biological importance, with an affinity for the estrogen receptor several-fold higher than that of *trans*-tamoxifen (Robertson et al. 1982).

Metabolites X and Z (*N*-demethyl-tamoxifen and *N*-didemethyl-tamoxifen) have low affinities for estrogen receptors and weak antiestrogenic activity (Jordan et al. 1983; Kemp et al. 1983). They are the metabolites found in the highest concentrations in blood during long term tamoxifen administration and may be detected by most of the HPLC methods developed for tamoxifen (table I).

The importance of other metabolites remains to be clarified. Metabolite A, formed by hydration of the ethylene double bond, was reported to be a minor metabolite in rhesus monkeys (Fromson et al. 1973a). It may be an artifact of the acid hydrolysis step used to isolate metabolites, since it has never been detected in other studies (Lyman & Jordan 1986). There are no data on the biological effects of this metabolite.

Metabolite C was found in the faeces of rhesus monkeys and dogs (Fromson et al. 1973a). The exact position of the methoxy group in this metabolite has not been established.

Metabolite D (catechol-3,4-hydroxy-tamoxifen), is present in the faeces of several species (Fromson et al. 1973a), but is not observed in humans during conventional treatment. However, after percutaneous application of [^{3}H]*trans*-4-hydroxy-tamoxifen, bypassing first-pass metabolism, the metabolite was detected (Mauvais-Jarvis et al. 1986). Metabolite D has a higher affinity than tamoxifen for the estrogen receptor, but its antiestrogenic activity *in vivo* is low (Robinson & Jordan 1987). This may be because the catechol derivative of tamoxifen is very unstable (Jordan 1984).

Metabolite E (tamoxifen without its amino-ethoxide side chain) is found as a minor metabolite in dog bile (Fromson et al. 1973a). It is the only metabolite with no antagonist properties, but acts as a pure agonist (Swain & Lippman 1990) and is reported to be present in minute quantities in plasma from patients (Murphy et al. 1987).

2.3.4 Plasma Pharmacokinetics

Peak plasma tamoxifen concentrations (C_{max}) are seen 3 to 7h after oral administration (Fabian et al. 1980). When oral [^{14}C]tamoxifen was administered, only about 30% of the radioactive material was present as tamoxifen when C_{max} was reached (Fromson et al. 1973b).

The $t_{½}$ of tamoxifen is 4 to 11 days (Adam et al. 1980b; Fromson et al. 1973b; Patterson et al. 1980). Assuming a bioavailability of 30%, as suggested by the results of Fromson et al. (1973b), plasma clearance (CL_p) could be about 1.2 to 5.1 L/h (Lien et al. 1990). Patients receiving tamoxifen 20mg twice daily achieve C^{ss} about 4 weeks after starting therapy (Patterson et al. 1980). On withdrawal of the drug, all metabolites showed first-order elimination with elimination rates approaching that of the parent drug (Lien et al. 1989). This suggests that the true elimination rate for most metabolites is similar to or could exceed that of tamoxifen and that serum concentrations of these metabolites are production-rate limited (Rowland & Tozer 1980).

Table II. Mean pharmacokinetic parameters and drug interactions of endocrine drugs used in the treatment of advanced breast cancer. See corresponding text for details and references

Drug	Dosage (mg/day)	F (%)	CL (L/h)	$t_{1/2}$ (h)	Vd (L)	Drug interactions
Gonadotrophin releasing hormone analogues						
Goserelin	3.6/4wk SC	NAO	2	5	14	NR
Buserelin	3.6/4wk SC	NAO	14	1.5	30[a]	NR
Antiestrogens						
Tamoxifen	20-40	Unknown	3.6[b]	170	>600[b]	Aminoglutethimide, warfarin?, phenobarbital?, MPA?
Toremifene	60-300	Unknown	5[c]	120	≈800[a,c]	NR
Droloxifene	20-100	Unknown	65[c]	25	>2000[a,c]	NR
Aromatase inhibitors						
Aminoglutethimide	250-1000	≈100	3	13	70	Tamoxifen, MPA, warfarin, dexamethasone, phenazone (antipyrine), digitoxin, theophylline
4-Hydroxyandro-stenedione	250-500 PO	Unknown	Unknown	3	Unknown	NR
	250/2wk-500/wk IM	Unknown	Unknown	170	Unknown	NR
Fadrazole	1-4	Unknown	37[c]	10	≈500[a,c]	NR
Pyridoglutethimide	400-1600	≈100	-[d]	-[d]	Unknown	NR
Progestins						
MPA	300-1000	Unknown	70[e]	60	>4000[f]	Aminoglutethimide
MA	40-160	Unknown	Unknown	14	Unknown	Aminoglutethimide

a Approximated from clearance and terminal half-life values in the literature.
b Assuming a bioavailability of 30%.
c Assuming a bioavailability of 100%.
d Nonlinear pharmacokinetics.
e Obtained after intravenous injection.
f Calculated from clearance values obtained after intravenous injections and terminal half-life reported in different studies.

Abbreviations: F = bioavailability after oral administration; CL = total body clearance; $t_{1/2}$ = elimination half-life; Vd = volume of distribution; MA = megestrol; NAO = not absorbed orally; NR = not reported; IM = intramuscular; PO = oral; wk = week; SC = subcutaneous; MPA = medroxyprogesterone.

The elimination $t_{1/2}$ of tamoxifen increases with successive doses (Adam et al. 1980b; Camaggi et al. 1985a). This may be due to reversible and partial inhibition of hepatic metabolism of the drug, a hypothesis supported by the fact that the terminal $t_{1/2}$ returns to initial values after a 145-day drug free interval (Adam et al. 1980b). Finding tamoxifen in the plasma of patients 8 weeks after the end of treatment and in tissues 1 year after (Lien et al. 1991a) suggests a long terminal $t_{1/2}$.

Metabolites X and Z are most often measured and present in the highest concentrations. Hydroxylated metabolites B and BX, of possible major biological importance, and the primary alcohol (metabolite Y), are also detectable (table I, fig. 2).

Several studies reported plasma concentrations of tamoxifen and metabolites Y, B, X and Z in women with breast cancer during long term treatment (Adam et al. 1980b; Brown et al. 1983; Daniel et al. 1979, 1981; Fabian et al. 1980; Kemp et al. 1983; Lien et al. 1989, 1990; Patterson et al. 1980). When conventional doses (20 to 40mg daily) are administered, mean tamoxifen C^{ss} values are

usually between 100 and 200 μg/L. The plasma concentration of metabolite X is usually about 50% higher than that of tamoxifen and that of metabolite Z is usually about 60 to 85% lower. Concentrations of the hydroxylated metabolites Y, B and BX are mostly between 5 and 20 μg/L plasma.

Having reached steady-state, the pharmacokinetics of tamoxifen appear to undergo little change, even after several years of treatment (Langan-Fahey et al. 1990). A dose- or plasma concentration-response relationship for tamoxifen has not been demonstrated (Lerner & Jordan 1990; Manni & Arafah 1981; Watkins 1988).

Data suggest that >98% of tamoxifen in plasma is bound to proteins, mainly albumin (Adam 1981; Lien et al. 1989; Sjöholm et al. 1979). This high protein binding may explain why tamoxifen occurs in very low, often undetectable, concentrations in the cerebrospinal fluid (Jordan et al. 1983; Lien et al. 1989; Noguchi et al. 1988).

Only anecdotal information is available concerning the influence of impaired renal and liver function on tamoxifen pharmacokinetics. One patient with impaired renal function had normal blood tamoxifen concentrations (Sutherland et al. 1984), while high plasma tamoxifen and metabolites B and X concentrations occurred in a patient with metastatic hepatic adenocarcinoma and liver obstruction (DeGregorio et al. 1989a).

2.3.5 Tissue Distribution

Several studies report tamoxifen tissue distribution in mice (DeGregorio et al. 1989; Fromson et al. 1973; Gottardis et al. 1988; Wilking et al. 1981), rats (Fromson et al. 1973; Major et al. 1976; Ruenitz & Bagley 1985) and other animals (Borgna & Rochefort 1981; Fromson et al. 1973). After intravenous [^{14}C]tamoxifen to spayed female mice, the radioactivity accumulated in the liver, bile, lungs and adrenal glands (Wilking et al. 1981). High concentrations of unmetabolised tamoxifen were detected in the lungs, adrenal glands and pancreas.

In rats, tamoxifen and metabolites B, BX, X and Z are found in most tissues in concentrations 8- to 70-fold higher than those in plasma (Lien et al. 1991a). The highest concentrations are in lung and liver; substantial amounts are also recovered from kidney and fat.

In humans, investigators found tamoxifen concentrations 10- to 60-fold higher than plasma concentrations in the liver, lung, brain, pancreas, skin and bone and in primary and metastatic breast cancer tissue (Daniel et al. 1981; DeGregorio et al. 1989; Fromson & Sharp 1974; Fromson et al. 1973b; Lien et al. 1991a,b; Milano et al. 1987; Robinson et al. 1991). The concentrations of tamoxifen and its metabolites in pleural, pericardial and peritoneal effusions may approach those in serum, with an effusion : serum ratio between 0.2 and 1. In saliva, concentrations of tamoxifen and metabolite X exceed the amounts of free drug in serum, suggesting active transport or trapping of these compounds in the salivary gland (Lien et al. 1989).

The apparent terminal volume of distribution (V_z) of tamoxifen in humans is large. Assuming a bioavailability of 30% after oral ingestion; V_z may be about 20 L/kg (Lien et al. 1989). This indicates a high tissue to plasma drug concentration ratio.

2.3.6 Drug Interactions

Drug interactions with tamoxifen metabolism have been reported *in vitro* and in animal studies, forecasting possible drug interactions in humans. A 105% increase in the rate of formation of metabolite X after phenobarbital induction of rat liver microsomes was observed (Ruenitz et al. 1984). There was a simultaneous 48% decrease in the rate of formation of metabolite B and a 100% decrease in the recovery of tamoxifen-*N*-oxide. The drug metabolism inhibitor, SKF 525-A, inhibited all these processes (Ruenitz et al. 1984).

Tamoxifen is a potent inhibitor of some mixed function oxidases *in vitro* (Meltzer et al. 1984) and inhibits its own metabolism (Adam et al. 1980b; Borgna & Rochefort 1981; Camaggi et al. 1985a). Life-threatening interactions between tamoxifen and warfarin have been reported (Lodwick et al. 1987; Ritchie & Grant 1989; Tenni et al. 1989). This suggests that tamoxifen may inhibit warfarin metabolism, but this has not been properly docu-

mented and the clinical relevance of tamoxifen enzyme inhibition is unclear.

Medroxyprogesterone acetate may reduce the C^{ss} of metabolite X (Camaggi et al. 1985b) and there is evidence of an interaction between tamoxifen and digitoxin, resulting in elevated plasma digitoxin concentrations (Middeke et al. 1986).

Antibiotics can interrupt the enterohepatic circulation of various drugs and hormones (Adlercreutz et al. 1976; Gorbach 1986). The intestinal microflora play an important role in intestinal reabsorption of many drugs and endogenous compounds due to their ability to hydrolyse conjugates and thereby promote reabsorption. Intestinal reabsorption of the tamoxifen-related compound diethylstilbestrol, which is excreted as a monoglucuronide in bile, is decreased in rats receiving antibiotics (Clark et al. 1969). Antibiotics that influence the intestinal flora will probably decrease intestinal reabsorption of tamoxifen and reduce serum concentrations of the drug, but this hypothesis has not been evaluated.

The serum concentrations of tamoxifen and its metabolites Y, B, BX, X and Z are markedly reduced after aminoglutethimide administration, corresponding to a mean increase in tamoxifen CL of 249% (Lien et al. 1990). Serum concentrations of most metabolites increased during aminoglutethimide treatment relative to tamoxifen concentrations.

2.4 Biochemical Actions of Toremifene

This drug is a novel antiestrogen with a chemical structure similar to tamoxifen, but with substitution of chlorine for a hydrogen atom (fig. 1). The chlorine atom is not lost during metabolism and so toremifene and tamoxifen have no common metabolites (Kangas 1990b).

Toremifene and its 4-hydroxylated metabolite both bind strongly to the estrogen receptor (Simberg et al. 1990) and both *in vitro* and *in vivo* investigations confirm that its antiestrogen activity is similar to that of tamoxifen (Kangas 1990b).

2.5 Clinical Experience with Toremifene

Several phase II trials with toremifene 60 to 300mg daily have been performed (Gundersen 1990; Hamm et al. 1991; Hietanen et al. 1990; Hindy et al. 1990; Modig et al. 1990; Valavaara et al. 1988). An objective response in 70 of 183 patients (38%) is comparable with that achieved by tamoxifen. The drug is currently undergoing several phase III studies (Pyrhonen 1990).

2.6 Clinical Pharmacokinetics of Toremifene

2.6.1 Drug Measurement

Different HPLC methods are currently available to measure toremifene and its major metabolites *N*-demethyl-toremifene and 4-hydroxytoremifene in plasma (Holleran et al. 1987; Webster et al. 1991). The main metabolites may be measured in urine by gas chromatography-mass spectrometry (GC-MS) methods (Watanabe et al. 1989).

2.6.2 Absorption

Radioactivity was excreted in similar patterns in the faeces and urine following intravenous and oral administration of radioactive toremifene to rats (Sipila et al. 1990). This suggests that the drug is fairly well absorbed, but it does not evaluate plasma bioavailability and the possibility of first-pass metabolism. Data suggest that both plasma and tissue drug concentrations are similar after oral and intravenous drug administration to rats and dogs (Kangas 1990b). In humans, the drug is absorbed similarly from tablets and solution and a linear dose-plasma AUC relationship has been reported (Anttila et al. 1990).

2.6.3 Metabolism and Excretion

After oral and intravenous radioactive toremifene to rats, most of the radioactivity was recovered from the faeces (Sipila et al. 1990). About 35 and 15% of the radioactivity is eliminated in the faeces and urine, respectively, during the first 7 days after oral administration of radioactive drug (Kangas 1990a).

Nine metabolites have been identified in the faeces of the rat (Sipila et al. 1990); the main metabolic pathways are 4-hydroxylation and *N*-demethylation. Several metabolites, such as the main human metabolite *N*-demethyl-toremifene, exhibit antiestrogenic activities (Kangas 1990c).

2.6.4 Plasma Pharmacokinetics

Toremifene and its 2 major metabolites, *N*-demethyl-toremifene and 4-hydroxy-toremifene, may be detected in the plasma of treated patients (Kohler et al. 1990; Wiebe et al. 1990). However, 4-hydroxy-toremifene has only been detected in plasma from patients receiving high dosages of the drug (Wiebe et al. 1990). The time to C_{max} (t_{max}) is between 1.5 and 6h from drug ingestion (Kohler et al. 1990; Tominaga et al. 1990). Terminal $t_{½}$ values of toremifene and its 2 major metabolites are about 5 to 6 days (Kohler et al. 1990; Tominaga et al. 1990; Wiebe et al. 1990). Plasma protein binding is more than 99% (Sipilia et al. 1988).

2.6.5 Tissue Distribution and Drug Interactions

Administration of [^{11}C]toremifene to rats resulted in high concentrations of radioactivity in brain and tumour tissue 50 min after injection (Kangas et al. 1989). Other results suggest high drug concentrations in several tissues, particularly the lungs (Kangas 1990b).

There are no published data suggesting interactions between toremifene and other drugs.

2.7 Biochemical Actions of Droloxifene

Droloxifene (3-hydroxy-tamoxifen) is a novel nonsteroidal antiestrogen with a structure similar to tamoxifen, but with a hydroxyl group introduced in the 3-position (fig. 1). Droloxifene binds to the estrogen receptor with a higher affinity than tamoxifen (Loser et al. 1985). *In vitro* and *in vivo* studies in animals show that the drug acts as a potent antiestrogen (Ahlemann et al. 1988; Loser et al. 1985).

2.8 Clinical Experience with Droloxifene

Preliminary results of phase II trials (Abe 1991; Bellmunt & Sole 1991; Dechenes 1990; Haarstad et al. 1991) with limited numbers of patients suggest droloxifene 100mg daily gives response rates comparable with those seen with other endocrine treatments.

2.9 Clinical Pharmacokinetics of Droloxifene

2.9.1 Drug Measurement

Droloxifene and its major plasma metabolite *N*-demethyl-droloxifene may be measured by HPLC (Huber & Stanislaus 1984).

2.9.2 Absorption

The drug is rapidly absorbed with the C_{max} occurring 1 to 4h after oral administration in rats (Huber & Stanislaus 1984). Similarly rapid absorption is seen in women with breast cancer (Stamm et al. 1986). The relative bioavailability of the drug in a tablet compared with a reference solution was more than 90% (Stamm et al. 1986). The absolute bioavailability of the drug in humans is not known.

2.9.3 Metabolism and Excretion

After oral radioactive droloxifene to rats, about 96% of the radioactivity was recovered in the faeces and only 1.3% in the urine (Huber & Stanislaus 1984). The main metabolites in humans are *N*-demethyl-droloxifene and glucuronides of *N*-demethyl-droloxifene and droloxifene (Stamm et al. 1986).

2.9.4 Plasma and Tissue Pharmacokinetics and Drug Interactions

Following ingestion of droloxifene 20mg, a C_{max} of about 25 μg/L is seen (Stamm et al. 1986). The terminal $t_{½}$ is about 25 to 27h (Grill & Pollow 1990; Stamm et al. 1986).

No data have been published on tissue distribution of droloxifene and its metabolites. Interactions between droloxifene and other drugs have also not been reported.

3. *Aromatase Inhibitors*

These drugs suppress postmenopausal estrogen synthesis by inhibiting the final step, the conversion (aromatisation) of circulating androgens into estrogens (Lønning et al. 1990; Santen et al. 1990). The androgen androstenedione is synthesised in the adrenal glands (about two-thirds of production) and the postmenopausal ovary (Vermeulen 1976). Aromatisation occurs in most peripheral tissues of the body, including organs such as the liver and within breast cancer tissue (Frisch el al. 1980; Matsumine et al. 1986; Miller et al. 1990; Perel & Killinger 1979; Schweikert et al. 1975, 1976; Smuk & Schwers 1977; Tilson-Mallett et al. 1983).

The prototype aromatase inhibitor is aminoglutethimide, which has now been used to treat breast cancer for 20 years (Lønning & Kvinnsland 1988). Aromatase inhibitors block estrogen production only in postmenopausal (or oophorectomised) women, whereas in premenopausal women ovarian estrogen synthesis is sustained by increased gonadotrophin release (Harris et al. 1982; Santen et al. 1980). Aminoglutethimide is the most extensively investigated aromatase inhibitor. Recently, several new aromatase inhibitors have been developed (Banting et al. 1989; di Salle et al. 1989; Hartmann et al. 1990; Nishino et al. 1989; Wouters et al. 1989); some are in phase I and II trials (Coombes et al. 1984; Haynes et al. 1991; Santen et al. 1989). Excepting 4-hydroxyandrostenedione (CGP 32349), the pharmacokinetic information and clinical data on the use of these drugs are limited.

3.1 Biochemical Actions of Aminoglutethimide

Aminoglutethimide inhibits several enzymes involved in adrenal steroid synthesis (Lønning & Kvinnsland 1988). However, due to increased ACTH excretion, adrenal steroid output is sustained and plasma androstenedione, the precursor of postmenopausal estrogen synthesis, may be elevated (Vermeulen et al. 1983). Aminoglutethimide suppression of plasma estrogens in postmenopausal women (Santen et al. 1982) with >90% inhibition of the peripheral conversion of androstenedione to estrone (Santen et al. 1978) introduced the term 'aromatase inhibition'. Recent findings suggest that the drug has a dual mechanism of action because it also enhances the metabolism of the important estrogen conjugate estrone sulfate (Lønning et al. 1987).

3.2 Clinical Experience with Aminoglutethimide

As a second- or third-line treatment of postmenopausal women with breast cancer, aminoglutethimide has an overall response rate of about 30% (Johannessen & Lønning 1991). Randomised trials reveal that the drug has a response rate similar to that of other endocrine treatments such as tamoxifen (Alonso-Munos et al. 1988; Harvey et al. 1982; Lipton et al. 1982; Smith et al. 1981) and high dose progestins (Canney et al. 1988; Lundgren et al. 1989b). Because of its side effects (Lønning & Kvinnsland 1988), effort has been made to develop less toxic aromatase inhibitors for clinical use.

3.3 Clinical Pharmacokinetics of Aminoglutethimide

3.3.1 Drug Measurement

Aminoglutethimide was previously measured spectrophotometrically (Murray et al. 1979). Several HPLC methods are now available to determine the drug and its major metabolite *N*-acetylaminoglutethimide in plasma (Adam et al. 1985; Menge & Dubois 1984; Robinson & Cornell 1983; Schance et al. 1984) and urine (Coombes et al. 1982). Aminoglutethimide is a racemic mixture of 2 optical enantiomers, with different biological effects (Graves & Salhanick 1979). Recently, an HPLC method to separate the enantiomers was published (Aboul-Enein et al. 1988). Aminoglutethimide is also measured by a specific multiple selected ion monitoring (SIM) method (Sirtori et al. 1988).

3.3.2 Absorption

Studies in rats and guinea-pigs show that the drug is well absorbed from the small intestine but poorly absorbed from the stomach (Eweiss et al. 1983). After oral [^{14}C]aminoglutethimide to women with breast cancer, 80 to 98% of the radioactivity was recovered in the urine within 72h (Lønning et al. 1985). Only about 1% of the drug is excreted unchanged in the faeces (Nicholls 1982). From knowledge of the CL, the presystemic clearance is estimated to be 2 to 8% (Lønning et al. 1985).

3.3.3 Metabolic Pathways and Metabolite Excretion

Several metabolites have been identified in the urine of rats and humans (Egger et al. 1982; Foster et al. 1984). A metabolite identified in human urine, hydroxy-aminoglutethimide, occurs only after multiple doses, suggesting production induced by long term treatment (Jarman et al. 1983). Only 1 metabolite, *N*-acetyl-aminoglutethimide, has been detected in human plasma (Adam et al. 1984; Coombes et al. 1980; Lønning et al. 1985).

An early study using a nonspecific photometric assay found 40 to 55% of oral aminoglutethimide was eliminated unchanged and 4 to 25% was excreted as *N*-acetyl-aminoglutethimide in the urine (Douglas & Nicholls 1965). More recent investigations using HPLC found 12 to 20% of the drug excreted unmetabolised in the urine and 3 to 7% excreted as *N*-acetyl-aminoglutethimide (Adam et al. 1984; Coombes et al. 1982). The total urinary output of aminoglutethimide and its known metabolites was quantified in 1 patient on different days (Goss et al. 1985). Between 40 and 75% of the dose was accounted for and the metabolism of aminoglutethimide is still incompletely understood.

3.3.4 Plasma Pharmacokinetics

After oral administration, C_{max} occurs within 0.5 to 4h in fasting patients (Lønning et al. 1985; Sirtori et al. 1988; Thompson et al. 1981). The pharmacokinetics of the enantiomers have not been evaluated separately.

Several investigators found a mean CL_p value of about 3 L/h (range 1.5 L/h to 6 L/h) and a mean $t_{1/2}$ of about 13 to 14h (range 6 to 24h) after single-dose administration (Adam et al. 1984; Lønning et al. 1985; Murray et al. 1979; Sirtori et al. 1988; Thompson et al. 1981). The plasma $t_{1/2}$ is significantly reduced during multiple-dose treatment (Adam et al. 1984; Lønning et al. 1985; Murray et al. 1979). Whether this is due only to autoinduction of drug metabolism (Murray et al. 1979) or to a reduction in Vd (Lønning et al. 1985) is controversial. One study (Miller et al. 1987) suggested plasma aminoglutethimide concentrations may decrease over time in patients on multiple-dose 1000mg daily but not among patients treated with 500mg daily. However, information about the time between administration and blood sampling is missing and these results conflict with the findings of others. A linear increase in plasma aminoglutethimide concentrations occurred over a 250 to 1000mg daily dosage range (Stuart-Harris et al. 1985). It seems unlikely that aminoglutethimide would cause significant autoinduction after 1000mg daily and yet not stimulate its own metabolism after 500mg daily, as suggested by Miller et al. (1987).

Aminoglutethimide is about 25% bound to plasma proteins (Lønning et al. 1985; Thompson et al. 1981). Assuming that the drug is totally absorbed, the mean Vd may be about 70 to 80L and thus may exceed the total body water content (Lønning et al. 1985; Thompson et al. 1981).

3.3.5 Tissue Distribution

Radioactive aminoglutethimide injected into mice resulted in high concentrations of radioactivity in such tissues as the nasal mucosa and adrenal cortex (Appelgren et al. 1985). After single doses, the drug appeared in the kidneys, liver and brain in concentrations similar to those seen in plasma, but concentrated in the adrenal glands (Ahmad et al. 1988).

Little is known about aminoglutethimide tissue distribution in humans. The erythrocyte : plasma concentration ratio is 1.4 to 1.7 (Thompson et al. 1981). In 1 case adrenal aminoglutethimide concentrations as high as 50 times that of the plasma concentration were found at autopsy (Cash et al.

1967) but the analysis used a nonspecific spectrophotometric method. There is no other information about aminoglutethimide concentrations in human tissues. The fact that the drug may have a Vd in excess of total body water (Adam et al. 1984; Lønning et al. 1985) suggests that peripheral tissue binding occurs.

3.3.6 Drug Interactions

Aminoglutethimide is a well known inducer of mixed function oxidases. Studies in rats demonstrate that the drug enhances the metabolism of hexobarbital and dicoumarol (Damanhouri et al. 1987a). While short term administration of high doses of aminoglutethimide may inhibit the mixed function oxidase in mice and rats (Damanhouri et al. 1987b) similar effects have not been reported in humans.

In a manner similar to barbital (Lønning & Kvinnsland 1988), aminoglutethimide enhances the metabolism of dexamethazone (Santen et al. 1974), phenazone (antipyrine), theophylline, digitoxin (Lønning et al. 1984) and warfarin (Lønning et al. 1986) in humans, the latter in a dose-dependent manner over dosages of 250 to 1000mg daily. In addition, aminoglutethimide suppresses plasma concentrations of progestins (Lundgren et al. 1990a; van Deijk et al. 1985) and tamoxifen (Lien et al. 1990), with a resultant need to increase the dosages of these drugs if they are going to be used concomitantly with aminoglutethimide.

3.4 Biochemical Actions of 4-Hydroxyandrostenedione

This drug is the most extensively investigated second generation aromatase inhibitor. Several androstenedione derivatives have been evaluated as aromatase inhibitors *in vitro* (Brodie et al. 1981a; Giudici et al. 1988), but 4-hydroxyandrostenedione is the only one tested in phase I and II trials. Apart from findings that it may inhibit 5-α-reductase in prostatic tissue (Brodie et al. 1989) it seems to act exclusively as an aromatase inhibitor without influencing other enzymes involved in steroid synthesis. *In vitro* and *in vivo* animal studies confirm that the drug is a much more potent aromatase inhibitor than aminoglutethimide (Brodie et al. 1986; Wing et al. 1985). It seems to act as a 'suicide inhibitor', irreversibly destroying the aromatase enzyme complex (Ayub & Levell 1987).

4-Hydroxyandrostenedione inhibits aromatisation *in vivo* in monkeys (Brodie & Longcope 1980) and humans (Reed et al. 1990) and it suppresses plasma estrone and estradiol in a manner similar to that of aminoglutethimide (Dowsett et al. 1989).

3.5 Clinical Experience with 4-Hydroxyandrostenedione

Clinical data including more than 370 patients are available (Coombes 1990; Höffken et al. 1990; Pickles et al. 1990). While the total response rate of 21% is somewhat lower than that seen during aminoglutethimide therapy, it might be due to differences in the patient groups included. The drug is currently undergoing phase III trials comparing its efficacy with high dose progestins and tamoxifen. Most patients received the drug by intramuscular injection (Coombes et al. 1990; Höffken et al. 1990), but it has also been given in an oral formulation (Cunningham et al. 1987), with no apparent difference in response rates between the 2 routes.

3.6 Clinical Pharmacokinetics of 4-Hydroxyandrostenedione

3.6.1 Drug Measurement

4-Hydroxyandrostenedione can be measured in plasma by RIA (Khubieh et al. 1990a) or isotope dilution mass spectrometry (Guarna et al. 1989). The drug and several metabolites may be quantified in urine using HPLC-MS (Poon et al. 1991).

3.6.2 Absorption

Few data on the absorption of 4-hydroxyandrostenedione are available. In rats, about 40% of an oral dose was recovered from the bile as glucuronides (Goss et al. 1986a). 20 to 45% of the drug (mean 35%) was recovered as 4-hydroxyandrostenedione glucuronide in the urine of patients with

breast cancer after oral administration (Dowsett & Lloyd 1990; Goss et al. 1986a).

The drug is available in a microcrystalline form for intramuscular injections (Goss et al. 1986b). Doses of 250 or 500mg every 1 to 2 weeks are usual (Dowsett et al. 1987, 1989; Goss et al. 1986b). The absorption ratio from the injection site has not been determined. 4-Hydroxyandrostenedione is not available for intravenous use and so the bioavailability of the drug has not been determined.

3.6.3 Metabolism and Excretion

When hepatocytes obtained from phenobarbital-treated rats were incubated with 4-hydroxyestrone, several metabolites, produced by oxidation and/or reduction, were formed (Foster et al. 1986). Two of these, 4-hydroxy-testosterone and 3β-hydroxy-5a-androstan-4,17-dione, have been detected in plasma from monkeys and rats receiving 4-hydroxyandrostenedione (Brodie et al. 1981b; Marsh et al. 1982).

4-Hydroxyandrostenedione glucuronide is the major metabolite in the urine of humans and bile of rats (Dowsett & Lloyd 1990; Goss et al. 1986a). An unidentified major metabolite has been observed in rat urine (Goss et al. 1986a). There is no significant excretion of unconjugated 4-hydroxyandrostenedione in humans (Goss et al. 1986a). Recently, 7 metabolites were identified in human urine (Poon et al. 1991) but the quantitative importance of the different metabolic pathways is unknown.

3.6.4 Plasma Pharmacokinetics

In rats, the C_{max} of 4-hydroxyandrostenedione occurs between 0.5 and 7h after oral intake (Khubieh et al. 1990b). In healthy males and female patients with breast cancer the median t_{max} was 1.5 to 2h (Dowsett & Lloyd 1990; Dowsett et al. 1989). A possible discrepancy between the t_{max} in rats and humans could be due to more pronounced enterohepatic circulation in the rat, with 4-hydroxyandrostenedione glucuronide found in high concentrations in the bile of this species (Goss et al. 1986a). The median terminal $t_{½}$ after oral administration was about 2h in healthy males (Dowsett & Lloyd 1990) and about 3h in women with breast cancer (Dowsett et al. 1989). After intramuscular injection, plasma concentrations decrease monoexponentially with a $t_{½}$ of 5 to 10 days (Dowsett et al. 1987, 1989). C_{max} values range from 10 to 30 μg/L after intramuscular administration and plasma estradiol levels seem to escape suppression when plasma drug concentrations decrease below 2 to 3 μg/L (Dowsett et al. 1987).

3.6.5 Tissue Distribution and Drug Interactions

So far there are no published data on tissue distribution of 4-hydroxyandrostenedione. The drug is not known to influence the disposition of other drugs and there are no data to suggest other drugs interfere with its disposition.

3.7 Biochemical Actions of Fadrazole (CGS 16949A)

Fadrazole belongs to a group of imidazole derivatives which act as aromatase inhibitors *in vitro*. It is the only compound of this class used to treat breast cancer so far (fig. 3). The drug is a potent inhibitor of the aromatase enzyme *in vitro* (Steele et al. 1987) and causes regression of certain hormone-dependent breast cancers in rodents (Schieweck et al. 1988).

In patients with breast cancer treated with fadrazole 1 to 2mg twice daily, 80 to 92% inhibition of the aromatase enzyme occurs (Lønning et al. 1991) and plasma estrone and estradiol levels are suppressed to the same extent as during treatment with aminoglutethimide (Dowsett et al. 1990b; Santen et al. 1989).

3.8 Clinical Experience with Fadrazole

Four phase I/II trials including a total of 138 evaluable patients have been conducted (Beretta et al. 1990; Harvey 1990; Lipton et al. 1990; Stein et al. 1990). The overall response rate was 14%; however, the drug was given in dosages from 0.3 to 10mg daily and recent results suggest that 2mg twice daily may be needed to optimise aromatase inhibition (Lønning et al. 1991).

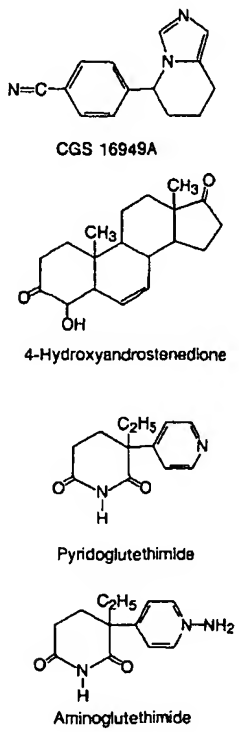


Fig. 3. Chemical structure of aromatase inhibitors currently used in breast cancer treatment.

3.9 Clinical Pharmacokinetics of Fadrazole

3.9.1 Drug Measurement

Currently, GC-MS (Ackermann & Kaiser 1989) and a liquid chromatography-MS (Kochak et al. 1990) method are available to measure plasma fadrazole concentrations. GC-MS is applicable to drug measurement in the urine as well.

3.9.2 Absorption, Metabolism and Excretion

There are no data published on the absorption of fadrazole in animals or on the metabolic pathways of fadrazole in animals or humans. C_{max} values were seen 1 to 4h following drug ingestion in patients with breast cancer (Kochak et al. 1990) but the fraction absorbed is unknown.

3.9.3 Plasma Pharmacokinetics

In women with breast cancer treated with fadrazole 2mg twice daily, C_{max} values from 3.5 to 14 μg/L were seen (Kochak et al. 1990). The same investigators reported that the drug has a terminal elimination $t_{½}$ of about 10.5h (range 5.7 to 29.3h) and a mean CL of about 37 L/h (range 15 to 78 L/h), assuming a bioavailability of 1.

3.9.4 Tissue Distribution and Drug Interactions

So far there are no data published on the distribution of fadrazole in animals or humans. Assuming the drug to be completely absorbed without any first-pass metabolism, Vd may be calculated from the data of Kochak et al. (1990). Thus, values of 200 to 1200L may be found, suggesting the drug could concentrate in body tissues.

No data on interactions between fadrazole and other drugs have been published.

3.10 Biochemical Actions and Clinical Experience with Pyridoglutethimide

Several aminoglutethimide analogues have aromatase inhibiting properties, some of which have no influence on adrenal steroid synthesis (Daly et al. 1986; Rowlands et al. 1988). One of these drugs, pyridoglutethimide, is used in breast cancer treatment (Dowsett et al. 1991; Haynes et al. 1991).

Pyridoglutethimide is similar in structure to aminoglutethimide (fig. 3), but, unlike the latter drug, has no inhibitory action on adrenal enzymes (Foster et al. 1985). Pyridoglutethimide suppresses plasma estradiol in postmenopausal patients with breast cancer (Dowsett et al. 1991; Haynes et al. 1991), but its influence on *in vivo* aromatisation has not been evaluated by use of tracer injections.

So far there are no data available on the clinical response rates to pyridoglutethimide.

3.11 Clinical Pharmacokinetics of Pyridoglutethimide

3.11.1 Drug Measurement

Pyridoglutethimide and its main metabolite may be measured in plasma by HPLC and in urine by combined thin layer chromatography and HPLC (Haynes et al. 1991; Seago et al. 1986).

3.11.2 Absorption

There is limited information on bioavailability of this drug in animals. Studies in rats revealed that about 15% of an ingested dose is excreted unchanged in the urine during 24h, while about 9% was excreted as the pyridoglutethimide-*N*-oxide, the only metabolite excreted at detectable concentrations (Seago et al. 1986). A study at the Royal Marsden Hospital found the drug to be well absorbed with similar AUC values when administered by intravenous and oral routes to patients with breast cancer (Dowsett et al. 1991).

3.11.3 Metabolism and Excretion

When rats were given parenteral pyridoglutethimide, about 15% of the dose was excreted unchanged in 24h urine and about 10% was excreted as the major metabolite *N*-oxide pyridoglutethimide (Seago et al. 1986).

Of a single 50mg oral dose administered to a volunteer, 5% was excreted unmetabolised and about 32% as the *N*-oxide in 24h urine (Seago et al. 1986). So far no metabolites except the *N*-oxide pyridoglutethimide have been detected in urine (Seago et al. 1986) or plasma (Haynes et al. 1991) of patients treated with pyridoglutethimide.

3.11.4 Plasma Pharmacokinetics

Following oral pyridoglutethimide, the drug and its main metabolite *N*-oxide pyridoglutethimide are detected in plasma of rats and humans (Dowsett et al. 1991; Haynes et al. 1991; Seago et al. 1986). In rats, plasma $t_{1/2}$ was about 6h (Seago et al. 1986). Pyridoglutethimide has interesting plasma pharmacokinetics in humans, with a disappearance rate that is best fitted by Michaelis-Menten kinetics (Haynes et al. 1991).

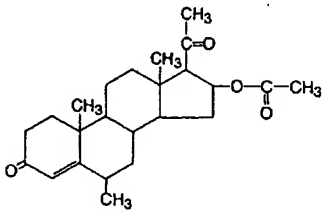
3.11.5 Tissue Distribution and Drug Interactions

So far there are no data published on tissue distribution of pyridoglutethimide.

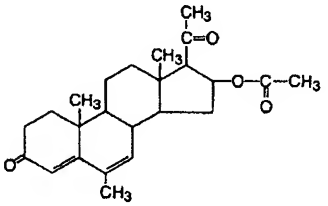
With its structural similarity to aminoglutethimide, pyridoglutethimide may enhance the metabolism of other drugs metabolised by hepatic mixed function oxidases. No such interactions have been recorded, but the possibility should be considered.

4. High-Dose Medroxyprogesterone and Megestrol

Progestins (progestagens or gestagens) are synthetic compounds chemically related to progesterone with progesterone agonist effects. Progestins were introduced in cancer treatment several years ago (Stoll 1967). Two classes have been developed for clinical use, the 19-norsteroids [including norethisterone (norethindrone) and *d*-norgestel] and the 17-OH-progesterone derivatives. The 2 progestins of interest in breast cancer treatment, medroxyprogesterone and megestrol, are 17-OH-progesterone derivatives known for more than 30 years (Babcock et al. 1958; Pagani et al. 1961; Sala et al. 1958). The only structural difference between the 2 drugs is a double bond between C_6 and C_7 positions in megestrol (fig. 4).



Medroxyprogesterone



Megesterol

Fig. 4. Chemical structure of medroxyprogesterone (I) and megestrol (II).

4.1 Biochemical Actions

Exact mechanisms by which progestins cause regression of breast cancer are unclear. Suppression of adrenal steroid synthesis (Alexieva-Figush et al. 1984; van Veelen et al. 1985) or estradiol receptor concentration (Tseng et al. 1975a), alterations in tumour hormone metabolism (Gurpide et al. 1977; Tseng et al. 1975b), enhancement of hepatic steroid metabolism (Gordon et al. 1971) and a direct cytotoxic influence on tumour cells (Allegra & Kiefer 1985; Iacobelli et al. 1982) have all been suggested. Progestins may influence some growth factors (Ewing et al. 1989; Horwitz et al. 1985) and suppress plasma estrone sulfate (Lundgren & Lønning 1990), mechanisms that warrant further investigation.

4.2 Clinical Experience

Progestins are used at 2 different dosages: 'low dose' and 'high dose' progestin therapy. Daily dosages of more than medroxyprogesterone 500mg or megestrol 60mg are defined as 'high dose'.

The first clinical studies exploring low doses of progestins (medroxyprogesterone 100 to 400mg daily or megestrol 30mg daily) resulted in response rates of <20% (Goldenberg et al. 1969; Segaloff 1967; Stoll 1967). A breakthrough in the use of progestins for breast cancer came with published results from treatment with medroxyprogesterone in high intramuscular doses (Pannuti et al. 1978). Medroxyprogesterone has been administered intramuscularly and orally, while megestrol is usually oral (Blumenschein 1983; Pannuti et al. 1979; Sedlacek & Horowitz 1984).

Different progestin regimens have been recommended (Beex et al. 1987; Paridaens et al. 1986); the overall response rate to both drugs of >30% (Sedlacek & Horwitz 1984) is similar to that of other endocrine treatments. Randomised studies show similar response rates with medroxyprogesterone, megestrol (Wander et al. 1987; Willemse et al. 1990) and such agents as tamoxifen (Ingle et al. 1982; Morgan 1985; Muss et al. 1985; van Veelen et al. 1986) or aminoglutethimide (Canney et al. 1988; Lundgren et al. 1989a). The progestins have also been successfully used as adjuvant therapy (Focan et al. 1989; Pannuti et al. 1988).

4.3 Clinical Pharmacokinetics

4.3.1 Drug Measurement

A number of methods to measure medroxyprogesterone and megestrol in plasma have been reported. Several RIA methods (Cornette et al. 1971; Hiroi et al. 1975; Royer et al. 1974; Shrimanker et al. 1978; Sletholt 1981; Smith et al. 1979; Victor & Johansson 1976), gas liquid chromatography (GLC) [Kaiser et al. 1974; Pannuti et al. 1982; Rossi et al. 1979], HPLC (Milano et al. 1982), enzymatic detection (Adlercreutz & Harkonen 1980) and GC-MS techniques (Adlercreutz et al. 1974; Phillippou & Frith 1980) have been published.

RIA methods use antibodies against the medroxyprogesterone-11-hydroxysuccinyl bovine serum albumin (BSA) conjugate or against the MPA-*O*-carboxymethyl-oxime-BSA conjugate. These antibodies have little cross-reactivity to natural steroid hormones, but some may cross-react with megestrol (Lundgren et al. 1986). Thus, the latter drug may be detected by the same RIA methods used for medroxyprogesterone (Adlercreutz et al. 1983; Utaaker et al. 1988).

4.3.2 Absorption

Low serum progestin concentrations, in particular medroxyprogesterone, are related to poor absorption. However, the exact oral bioavailability of progestins is unknown, as no studies have compared plasma AUC following oral and intravenous administration.

The significant difference in oral bioavailability between the 2 progestins is illustrated by the finding that megestrol 160mg daily seems to produce a plasma drug concentration 2- to 3-fold higher than that achieved after medroxyprogesterone 1000mg daily (Lundgren & Lønning 1990; Lundgren et al. 1989b, 1990b).

When the AUC after oral medroxyprogesterone was compared with those after intraperitoneal and intramuscular injections, the relative oral bioavail-

ability was only 0.2 to 17.4% (Camaggi et al. 1985c). However, after oral [^{14}C]megestrol, 55 to 80% of the radioactivity was recovered in the urine (Cooper & Kellie 1968). Thus, megestrol may be well absorbed, but possibly subject to first-pass metabolism.

4.3.3 Metabolism and Excretion

Development of synthetic progestins with 6-methyl and 17-acetyl groups was intended to reduce metabolism of the compounds. Biological activity of the metabolites of medroxyprogesterone and megestrol has not been investigated.

The metabolism of these 2 agents varies between species (Fotherby & James 1977). When [^{3}H]medroxyprogesterone is given parenterally to rabbits and hamsters, 30 to 40% of the radioactivity is found in the urine and about 10% in the faeces (Cooper & Kellie 1968; Zbuzkova & Kinci 1970). However, animals such as rats, sheep and swine excrete more radioactivity in the faeces than in urine (Kinci et al. 1970; Ogilvy 1965). In humans, 25 to 50% of the radioactivity is excreted in the urine and about 5 to 10% in the stools following intravenous administration of [^{3}H]medroxyprogesterone (Fotherby et al. 1968; Fukushima et al. 1979; Slaunwhite & Sandberg 1961; Utaaker et al. 1988). Thus, it is possible to account for only about half the radioactivity; the fate of residual radioactivity is unknown.

The major metabolite in urine after intravenous medroxyprogesterone is a glucuronide, presumably the 3-enol form. Less than 3% of the dose is excreted as unconjugated medroxyprogesterone in humans (Pannuti et al. 1984; Slaunwhite & Sandberg 1961).

There have been 15 urinary metabolites detected in the urine of patients receiving medroxyprogesterone (Fukushima et al. 1979; Sturm et al. 1991). Metabolism is by hydroxylation in 2-, 6- and 21-positions, reduction, demethylation and combinations of the different reactions.

After oral [^{14}C]megestrol, 56 to 78% of the radioactivity was found in the urine and 8 to 30% in the faeces (Cooper & Kellie 1968). Total recovery of radioactivity was 83 to 95%.

Considering the metabolic fate of megestrol, 3 metabolite glucuronides (megestrol hydroxylated in the 2-position, 6-methyl-position, or both) have been identified from human urine (Cooper & Kellie 1968; Martin & Adlercreutz 1977). They account for 5 to 8% of the radioactivity injected (Cooper & Kellie 1968).

4.3.4 Plasma Pharmacokinetics

The CL of medroxyprogesterone in relation to bodyweight was remarkably similar in rats (29 L/day/kg bodyweight), monkeys (26 L/day/kg) and dogs (45 L/day/kg) [Gupta et al. 1977]. In humans, the metabolic clearance rate of medroxyprogesterone after intravenous bolus administration is between 27 and 70 L/h (Gupta et al. 1979; Utaaker et al. 1988), corresponding to the rates in rats and monkeys. However, in the latter 2 studies blood samples were collected over a period of 6h only. Thus, the terminal rate constant may be overestimated, resulting in an underestimation of AUC and overestimation of CL_p.

The initial volume of distribution (V_0) is about 4 to 8L in humans (Gupta et al. 1979; Utaaker et al. 1988). After an intravenous bolus injection the disappearance of medroxyprogesterone from plasma is best fitted to a 3-exponential model over the first 6h after injection (Utaaker et al. 1988). However, it is mandatory to obtain blood samples over a sufficient interval to calculate the terminal rate constant. Some studies reported a mean terminal $t_{½}$ of medroxyprogesterone of about 7 to 12h and a $t_{½}$ for megestrol of about 14h after oral (Adlercreutz et al. 1983) or intramuscular (Stockdale et al. 1987) administration. However, in those studies blood sampling was performed for 24h only. Pannuti et al. (1982) reported a mean terminal $t_{½}$ for medroxyprogesterone of about 60h in patients in whom blood samples were obtained over a period of 146h after single oral doses.

The t_{max} of medroxyprogesterone is 2 to 5h after an oral dose and a linear dose/C_{max} relationship has been reported (Løber et al. 1981; Stockdale et al. 1987). Similarly, the C_{max} of megestrol occurs 2 to 5h after drug intake (Gaver et al. 1985).

Single medroxyprogesterone 1000mg doses may

produce C_{max} values of 40 to 120 μg/L; single megestrol 160mg doses result in values of 90 to 140 μg/L. Plasma AUC values after single medroxyprogesterone 1000mg doses or megestrol 160mg were 230 to 900 μg/L · h and 1860 to 2425 μg/L · h, respectively (Gaver et al. 1985; Pannuti et al. 1982; Stockdale et al. 1987). Medroxyprogesterone is slightly bound to albumin and does not bind specifically to other plasma proteins (Mathrubutham & Fotherby 1981). To our knowledge, no study of plasma protein binding of megestrol has been conducted.

Following oral medroxyprogesterone or megestrol, glucuronidated metabolites appear in high concentrations in the plasma (Adlercreutz et al. 1983). Some metabolites may cross-react with the antibody in RIA. Thus, it is important to separate free drug from conjugates by organic extraction of the samples. If RIA is performed after organic extraction megestrol and medroxyprogesterone concentrations are similar to those found with specific HPLC or GC-MS methods (Martin & Adlercreutz 1977; Milano et al. 1982).

It is not clear whether there are certain drug concentration 'thresholds' for medroxyprogesterone and megestrol to achieve an optimal response rate. One study suggested that patients with mean plasma drug concentrations higher than 100 to 150 μg/L may have a better response rate than patients with lower drug concentrations (Johnson et al. 1986), while others have not found a drug concentration/response relationship (Beex et al. 1987; Hedley et al. 1985; Jakobsen et al. 1986; Lundgren et al. 1989b). One study suggested a similar response rate to medroxyprogesterone 300 and 1000mg daily (Gallagher 1987), but too few patients were included to address the question properly in statistical terms.

4.3.5 Tissue Distribution

Medroxyprogesterone and megestrol seem to concentrate in the liver and breast tumour tissue in rodents (Kinci et al. 1970). Autopsies in humans suggest that medroxyprogesterone is concentrated in the small intestine, colon and adipose tissue (Pannuti et al. 1984). Considering the high CL_p values but long $t_{1/2}$ these findings indicate the Vd values for megestrol and medroxyprogesterone are extremely high, suggesting a high tissue C^{ss} for both drugs.

4.3.6 Drug Interactions

Aminoglutethimide suppresses plasma medroxyprogesterone and megestrol concentrations when the drugs are given together (Lundgren et al. 1990a; van Deijk et al. 1985). Progestins may increase plasma warfarin concentrations (Lundgren et al. 1986) and medroxyprogesterone seems to reduce the plasma metabolite X concentration in patients receiving tamoxifen (Camaggi et al. 1985).

5. Conclusions

Endocrine therapy has an important place in the treatment of advanced breast cancer. Drugs such as tamoxifen and aminoglutethimide have been used to treat this disease for more than 20 years and thousands of new patients every year receive treatment with these drugs and with high dose progestins. Several new antiestrogens and aromatase inhibitors have been introduced for clinical use and while many studies have been conducted to evaluate the plasma pharmacokinetics of these drugs, we still lack essential information about basic pharmacokinetic parameters. Information about tissue distribution and metabolite pharmacokinetics of most of these drugs is incomplete. The importance of drug interactions is underlined as they may be relevant to clinical considerations of which drugs may be used together.

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Review

Metabolites of tamoxifen in animals and man: identification, pharmacology, and significance

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Keywords: antiestrogens, estrogen receptor, monohydroxytamoxifen, pharmacology of tamoxifen, tamoxifen metabolites

Summary

Over the past decade, the non-steroidal antiestrogen tamoxifen has gained general acceptance for the palliative treatment of breast cancer. Although there has been much interest in the pharmacology of tamoxifen, our knowledge of its metabolism in laboratory animals and patients is incomplete and the precise mechanism of action within target tissue and breast tumor cells is unknown. This review briefly describes the pharmacology of tamoxifen in various laboratory species and patients. Several metabolites of tamoxifen are known and their relative potencies as estrogens and antiestrogens are compared with the parent compound. Apart from monohydroxytamoxifen, none of tamoxifen's metabolites are more potent antiestrogens, but a metabolite in the dog, Metabolite E, is fully estrogenic. Routine assays (tlc, HPLC, glc/ms) are available to detect tamoxifen, N-desmethyltamoxifen, monohydroxytamoxifen, and a newly identified metabolite, designated Metabolite Y, in biological fluids. Continuous therapy with tamoxifen (10 mg bid) produces steady-state levels (100-200 ng/ml serum) within 4 weeks. Levels of N-desmethyltamoxifen are often up to twice the levels achieved with tamoxifen, while levels of monohydroxytamoxifen and Metabolite Y are below 10 ng/ml. Although monohydroxytamoxifen has a high binding affinity for the estrogen receptor, the metabolic activation of tamoxifen is an advantage rather than a requirement for antiestrogenic activity. The action of tamoxifen in vivo is the net result of the individual actions of the parent compound and its metabolites competing for the occupation of receptors within target tissues and tumors.

Introduction

The non-steroidal antiestrogens are a large group of compounds whose structures are based on, or closely related to triphenylethylene (Fig. 1). Initially, antiestrogens were developed as potential antifertility agents (see 1 for a review), but when clinical trials with clomiphene (Fig. 1) demonstrated induction of ovulation in subfertile women (2), enthusiasm waned. At that time (1960s) there was much interest in endocrine manipulation to achieve objective remission in sensitive breast cancers. The presence of the estrogen receptor in tumors that respond to endocrine therapy, focused attention on the primary importance of estradiol for the homeostasis and growth of breast tumors (3). It was, therefore, only natural that the known activity of antiestrogens to block estradiol binding

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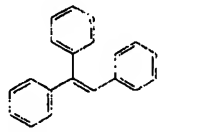
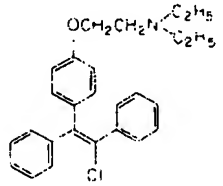
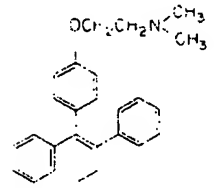


Triphenylethylene



Enclomiphene (trans)
(Clomid® is a 1:1 mixture of
cis and *trans* isomers)



Tamoxifen (Nolvadex®)

Fig. 1. Structural formulae of non-steroidal estrogens and antiestrogens.

to the estrogen receptor, should stimulate the organization of clinical trials to evaluate these drugs as a logical approach to breast cancer therapy. The results of the early trials were encouraging (4-10). However, only tamoxifen (Fig. 1) was found to have a low incidence of side effects (11), which made it the antiestrogen of choice for further evaluation.

Tamoxifen is now used routinely for the treatment of advanced breast cancer. The objective response rate achieved (approximately 30% of a patient population) is similar to other endocrine therapies (12-15). However, tamoxifen has a high degree of patient acceptability, and as yet there are few reports of serious side effects (16, 17). For this reason, tamoxifen is being evaluated as an adjuvant therapy following mastectomy. Early results are encouraging for certain categories of patients (18, 19). Nevertheless, comparisons of different treatment regimens and periods of treatment are required before making any final conclusions about the value of this drug.

The widespread clinical use of tamoxifen has increased interest in its pharmacology and mechanism of action. It is established that tamoxifen inhibits the binding of estradiol to the estrogen receptor, but the precise subcellular events that lead to an inhibition of estrogen action are at present unknown. It is not within the scope of this article to review all the possible mechanisms of action of the antiestrogens, but the reader may wish to consult some recent reviews (20-23) to obtain this information.

In this review the pharmacokinetics and metabolism of tamoxifen will be considered. However, the reader should appreciate that the pharmacology of tamoxifen is extremely complex. There are profound, and as yet unexplained, species differences: for example, tamoxifen is estrogenic in some species and antiestrogenic in others. Furthermore, opposing actions of the drug are often observed in the same species. In the light of this information it is perhaps naive to suggest that the tamoxifen molecule alone is responsible for all of the actions of the drug. Conversely, it may be an oversimplification to suggest that tamoxifen is a pro-drug and only the metabolites are active in vivo. The tissue response to any drug in vivo is the net result of the individual actions of the parent drug and its pharmacologically active metabolites. In the case of tamoxifen, inhibition of estrogen action will depend upon the local concentration of tamoxifen and its metabolites in the tumor and their relative binding affinities for the estrogen receptor. For this reason, the unusual pharmacological actions of tamoxifen are first briefly reviewed, and the possible importance of metabolism for the efficacy of tamoxifen as an antitumor agent will then be considered. This will be done by dissecting out what is known about the pharmacology of individual metabolites.

Pharmacology of tamoxifen

Tamoxifen (ICI 46,474: *trans* 1-(4-β dimethylaminoethoxyphenyl) 1,2-diphenylbut-1-ene) is described as a full estrogen in mouse uterine and

vaginal assays (24–26). However, high doses of tamoxifen administered subcutaneously can cause the vagina to become refractory and unable to respond to administered estradiol (27, 28). Similarly, prolonged administration of tamoxifen to mice causes the uterus to respond as if the drug was a partial agonist with potential antiestrogenic properties (29). In complete contrast, tamoxifen is antiestrogenic and exhibits no estrogenic effects in the chick oviduct (30) and liver (31).

The complex pharmacology of tamoxifen is perhaps best illustrated in the rat and human, where well defined estrogenic and antiestrogenic effects occur in different organs or cells of the same species. In the rat, tamoxifen inhibits estrogen-stimulated rises in uterine wet weight (24, 26) and estrogen-stimulated vaginal cornification (24), and partially decreases the estrogen-stimulated increase in serum prolactin levels (32). Similarly, tamoxifen inhibits the initiation (33), promotion (34), and continued growth (35–37) of hormone-dependent dimethylbenzanthracene (DMBA)-induced rat mammary tumors. Tamoxifen is also active in reducing the number of rat mammary tumors induced by whole body irradiation (38). In contrast, tamoxifen has clear-cut estrogenic actions in the rat. There is a partial increase in uterine wet weight (24), a full stimulation of uterine luminal epithelial cells (39, 40), progesterone receptor synthesis (41, 42), and ornithine decarboxylase activity (43). Circulating levels of renin substrate are increased equally by estradiol and tamoxifen (44, 45), and prolonged treatment with tamoxifen completely lowers the circulating level of luteinizing hormone (LH) in the ovariectomized rat (46).

A similar mixture of antiestrogenic and estrogenic effects has been reported in patients during the use of tamoxifen for breast cancer therapy. Regression of breast tumor growth with tamoxifen is considered to be the result of an inhibition of estrogen action by blocking estrogen binding to the receptor (47–54). Be that as it may, tamoxifen has a direct inhibitory action on the growth of MCF-7 breast cancer cells in culture, and this can be reversed by the addition of estradiol to the media (55–57). Several of the effects of tamoxifen noted in patients can be considered to be antiestrogenic actions. The estrogen-stimulated increase in mid-cycle prolactin in premenopausal women is reduced during tamoxifen therapy (58), and continuous tamoxifen therapy blunts the release of prolactin by thyroid stimulating hormone (59). Similarly, the elevated levels of circulating estradiol seen in premenopausal women on tamoxifen therapy (60) could be the result of an inhibition of an estrogen-mediated negative feedback mechanism in the hypothalamo-pituitary axis. This action would increase gonadotropin output, and consequently steroidogenesis. Nevertheless, a direct action of tamoxifen on the ovary can not, as yet, be excluded. In contrast, tamoxifen produces estrogen-like changes in the profile of plasma proteins (61), decreases antithrombin III (62), and increases the levels of circulating steroid hormone binding globulin (SHBG) (63). A partial decrease in the circulating level of LH has been noted (64), and estrogen-like changes in vaginal epithelium are often observed during prolonged therapy with tamoxifen (65). Finally, an estrogen-like increase in the level of progesterone receptors has been reported with tamoxifen in breast (66) and uterine (67) carcinoma tissue in vivo as well as in MCF-7 breast cancer cells in vitro (68).

Detection of metabolites

Early studies by Fromson and co-workers (69) described the metabolism of ^{14}C-labelled tamoxifen in rats, mice, rhesus monkeys, and dogs. Several metabolites of tamoxifen were identified by a combination of thin layer chromatography (tlc) and gas liquid chromatography/mass spectrometry (glc/ms). Unfortunately, these experiments were designed only to establish excretion rates for the drug in different species, rather than to evaluate critically differences in metabolic profiles that might account for tamoxifen's unusual pharmacology. Nevertheless, it was established that tamoxifen has a long biological half-life in all species studied, with 80–90% of the radioactive dose eliminated in the feces over a 10–20-day period. Biliary excretion is the major route of drug elimination, and enterohepatic recirculation was established for the rat and dog.

In a related study (70), ^{14}C-labelled tamoxifen

(0.3 mg/kg) was administered orally to four patients to determine pharmacokinetics. A peak level of radioactivity in the blood was reached within 4-7 hr, but after an initial half-life of 7–14 hr the secondary decay of radioactivity was greater than 7 days. Unlike the investigation in animals (69), the study in patients relied only on tlc of plasma extracts against synthetic standards to identify metabolites. Using this methodology, a monohydroxylated metabolite of tamoxifen (Metabolite B, monohydroxytamoxifen, ICI 79,280) was reported to be the major non-conjugated metabolite in man. However, recent improvements in analytical techniques have shown that this conclusion was incorrect. Monohydroxytamoxifen is only a minor metabolite in man; N-desmethyltamoxifen (ICI 55,548) is the major metabolite of tamoxifen in patients (71). The structures of the confirmed metabolites of tamoxifen in various species, including man, are summarized in Fig. 2. It should, however, be pointed out that another metabolite, designated Metabolite F (Fig. 3), was apparently identified in rat bile (69) and patient serum (70), but there are no reports of the pharmacology of the metabolite, or indeed any subsequent reports confirming its identification as a metabolite of tamoxifen.

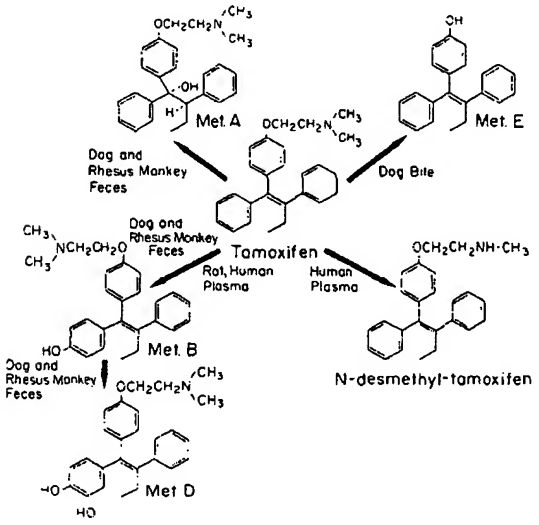
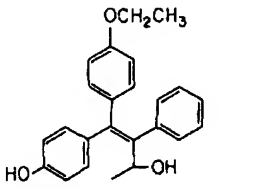


Fig. 2. Metabolites of tamoxifen in various species.



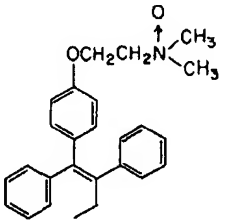


Fig. 3. Metabolites of tamoxifen.

There has been little use of established techniques in vitro to study the metabolism of tamoxifen. It almost seems obvious that the preparation of liver microsomes from different species could provide invaluable information about the relative importance of different metabolic routes for tamoxifen in a particular species. Recently, tamoxifen N-oxide (Fig. 3) was identified as a metabolite of tamoxifen using a liver microsomal preparation from phenobarbitone-treated male rats (72). In this study, the authors suggested that tamoxifen N-oxide (6%) could be a precursor of N-desmethyltamoxifen, which was the major metabolite (20%). Apparently there was some difficulty in being able to identify monohydroxytamoxifen as a minor metabolite (1.5%). Clearly this whole approach will provide much useful information in the future when different species are compared and the importance of P450 enzyme induction and drug interactions are adequately documented.

The widespread use of tamoxifen for the treatment of breast cancer has stimulated efforts to develop routine assays to quantitate the parent compound and metabolites in biological fluids. The published methods are compared in Table 1. The tlc and high performance liquid chromatography (HPLC) methods depend for their sensitivity upon the conversion of triphenylethylenes to

Table 1. Comparison of the assay methods available to measure the concentration of tamoxifen and its metabolites in biological fluids.

Assay method		Compound identified (Ref)	Sensitivity (per ml)	Internal standard[b]
tlc	1.	Tamoxifen (73)	2.5 ng	A
	2.	N-desmethyltamoxifen (74)	2.5 ng	A
HPLC	1.	Tamoxifen, monohydroxytamoxifen (75)	1 ng	none
	2.	Tamoxifen, N-desmethyltamoxifen, monohydroxytamoxifen (76)	0.1 ng	none
glc/ms	1.	Tamoxifen (77)	nd[a]	B
	2.	Tamoxifen,	1 ng	C
		monohydroxytamoxifen (78)		D
	3.	Tamoxifen,	—	cis/trans C
		monohydroxytamoxifen,	—	cis/trans D
		N-desmethyltamoxifen (79)	nd	E

[a] nd = not described

[b] Chemical names of internal standards

A 1-(4-β dimethylaminoethoxyphenyl) 2,2 diphenylacrylonitrile (ICI 99,311)

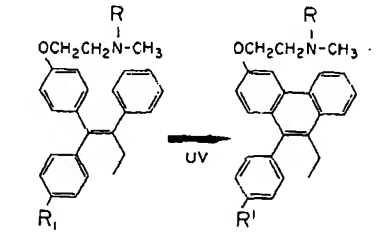
B *trans* 1-(4-β dimethylaminoethoxyphenyl) 1,2 diphenyl prop-1-ene

C *trans* 1-(4-β dimethylaminoethoxyphenyl) 1,2 diphenyl pent-1-ene

D *trans* 1-(4-β dimethylaminoethoxyphenyl)-1-(4-hydroxyphenyl) 2 phenyl pent-1-ene

E *cis/trans* 1-(4-β methylaminoethoxyphenyl) 1,2 diphenyl pent-1-ene

fluorescent phenanthrene derivatives by UV light. The reaction for tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen is shown in Fig. 4. Recently, we validated a new and improved HPLC method for the assay of tamoxifen and its metabolites (Brown, Bain and Jordan, in preparation). Unlike the published HPLC methods (75, 76) that have no internal standard and convert triphenylethylenes to phenanthrenes before chromatography, our methodology uses ICI 99,311 or Metabolite E (depending upon the column used to detect different metabolites) as internal standards and post-column UV activation and fluorescence detection. Although we have identified new metabolites with this methodology (see later), there is no doubt that glc/ms is the most specific assay method (79). However, this latter technique suffers from the disadvantage of requiring expensive equipment not generally available in routine assay laboratories.



	R	R'
Tamoxifen	CH_3	H
Desmethyltamoxifen	H	H
Monohydroxytamoxifen (Met B)	CH_3	OH

Fig. 4. Conversion of tamoxifen and metabolites to fluorescent phenanthrenes by UV activation.

Each of the assay procedures has been used to monitor the pharmacokinetics of tamoxifen, and in some cases its metabolites, in patients. Overall, the studies confirm that tamoxifen has a long biological half-life and readily accumulates to steady-state levels on repeated administration. A single oral dose of 10 mg tamoxifen produces peak serum levels of tamoxifen of 20–30 ng/ml within 3–6 hrs, but patient variation is very large (80). Nevertheless, continuous therapy with either 10 mg bid (73) or 20 mg bid (80) tamoxifen produces a steady state in serum within 4 weeks. In general, the serum levels of N-desmethyltamoxifen are up to twice the levels

of tamoxifen, but again the patient to patient variation is very great. This is illustrated in Figs. 5a and b to show the serum levels of tamoxifen and N-desmethyltamoxifen in patients receiving 10 mg bid for breast cancer therapy. The patient variation is apparent (Fig. 5a vs Fig. 5b). It should also be pointed out that monohydroxytamoxifen was not detectable using the tlc methodology, confirming previous reports (73). However, at a dosage regimen of 20 mg bid tamoxifen, serum levels of 10 ng monohydroxytamoxifen/ml have been reported (78, 79, 81).

Attempts to correlate early serum levels of tamoxifen or N-desmethyltamoxifen with the responsiveness of the breast tumor have not been successful (82). The hormone sensitivity of the tumor rather than the adsorption of the drug seems to be of fundamental importance (49–54). Nevertheless, it has been suggested that the administration of a large loading of tamoxifen might cause sensitive tumors to regress at an earlier time (80). No data has yet been presented to suggest that this approach can be an advantage over normal therapeutic regimens.

Patients taking 20 mg tamoxifen bid achieve a steady state for tamoxifen at 4 weeks and for N-desmethyltamoxifen at 8 weeks. These data have been used to calculate approximate biological half-lives of 7 and 14 days for tamoxifen and N-desmethyltamoxifen respectively (82). Tamoxifen can be detected in serum for many weeks after therapy is stopped (80). In Fig. 6 the levels of tamoxifen and N-desmethyltamoxifen can be seen to decrease slowly after the 10 mg bid therapy with tamoxifen is stopped. The clinician should be concerned to ensure that sufficient time has elapsed between the cessation of tamoxifen therapy and attempts to determine the estrogen receptor status of a tumor mass. Clearly an estrogen receptor positive result can be viewed with confidence, but an estrogen receptor negative result might result from residual binding ligands sequestered within the tumor. A delay for up to 6 weeks may be necessary to ensure excretion of estrogen receptor binding ligands before conventional receptor assays can be attempted.

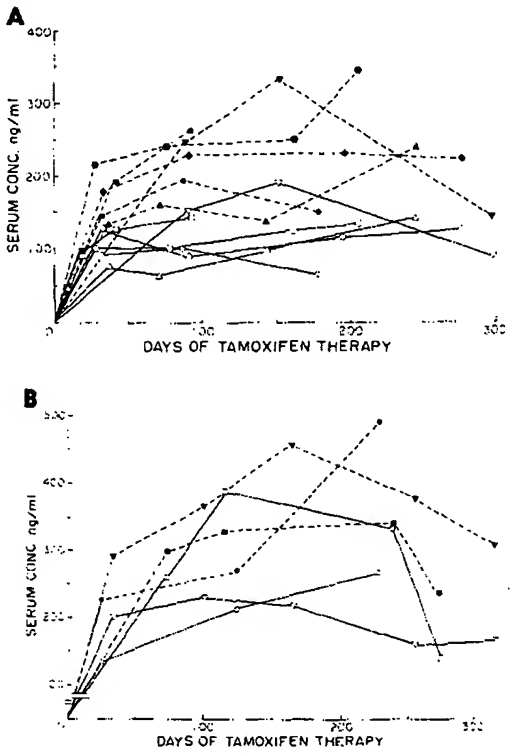


Fig. 5. The serum levels of tamoxifen (solid lines) and N-desmethyltamoxifen (broken lines) in breast cancer patients receiving tamoxifen 10 mg bid. Each symbol (open or closed) represents a separate patient. The assay method (tlc) was an adaptation of that described by Adam and co-workers (73).

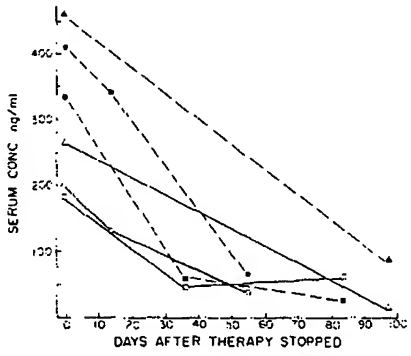


Fig. 6. The serum levels of tamoxifen (solid lines) and N-desmethyltamoxifen (broken lines) in 3 patients at various times after stopping tamoxifen therapy (10 mg bid). The symbols (open or closed) represent individual patients. The assay method (tlc) was an adaptation of that described by Adam and co-workers (73).

Recently we described (83) the preliminary identification of another metabolite of tamoxifen observed in serum of patients receiving high dose tamoxifen therapy (150 mg bid). The type of result that attracted our attention is illustrated in Fig. 7. Normal therapy with 10 mg bid tamoxifen produces a chromatogram of serum extracts with N-desmethyltamoxifen and tamoxifen peaks. Chromatography of serum extracts of a patient receiving 150 mg bid shows very large N-desmethyltamoxifen and tamoxifen peaks but also an unidentified peak that runs just ahead of the internal standard (ICI 99,331). The unknown compound co-chromatographs on tlc and HPLC systems with Metabolite E (tamoxifen without the side chain), but the mass spectrum of purified material is not the same as authentic Metabolite E. The parent ion signal of the unknown metabolite indicates a molecular weight of 344, which is consistent with the structure shown in Fig. 8. The compound has been given the designation Metabolite Y. Authentic synthetic Metabolite Y, obtained from ICI Ltd. Pharmaceuticals Division, Macclesfield, Cheshire, co-chromatographs with the unknown metabolite in patients using the tlc system. We further characterized the unknown compound by developing an HPLC system to retain all amine-containing triphenylethylenes. The unknown metabolite and Metabolite Y co-chromatograph in this system and are well separated from authentic Metabolite E, which we now use as a routine internal standard in this HPLC system (Fig. 9).

In summary, a whole spectrum of metabolites of tamoxifen have been identified, all of which could play a role in the subcellular pharmacology of the

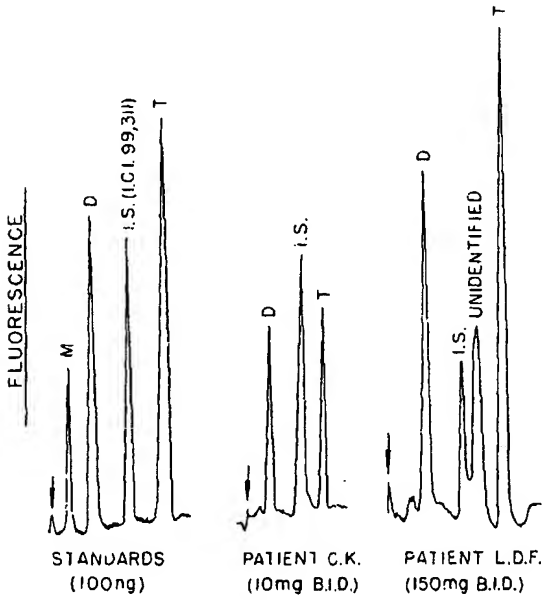


Fig. 7. Fluorescent scans of thin layer chromatograms of serum extracts for normal dose (10 mg bid) and high dose (150 mg bid) patients taking tamoxifen. The chromatography was run from left to right (the arrow indicates the origin). The authentic standards were monohydroxytamoxifen (M), N-desmethyltamoxifen (D), and tamoxifen (T), using ICI 99,311 as an internal standard (IS) to determine the efficiency of extraction. The method used was an adaptation of that described by Adam and co-workers (73).

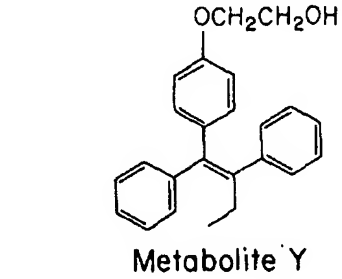


Fig. 8. Proposed structure of the new metabolite of tamoxifen identified in patient plasma.

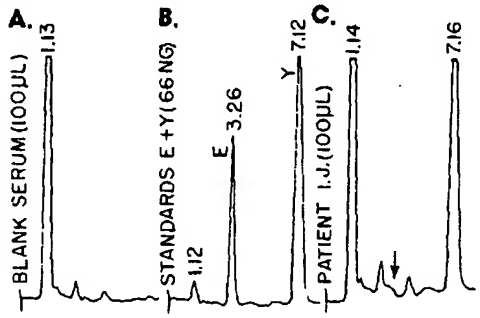


Fig. 9. High performance liquid chromatography (HPLC) of: (A) extract of blank patient serum containing no fluorescent peaks other than the void volume (1.13 min); (B) injected standards of Metabolite E (3.26 min) and Metabolite Y (7.12 min); and (C) extract of serum from a patient who was taking tamoxifen 150 mg (bid) for 6 months. No Metabolite E was detected (arrow) but Metabolite Y (7.16 min) was present.

drug. It is, therefore, important to consider the biological properties of individual metabolites in order to appreciate fully the complex interactions of different binding ligands at the estrogen receptor.

Pharmacology of the metabolites of tamoxifen

During the past 5 years there has been increased interest in the pharmacology of tamoxifen's metabolites. Much of this interest is a direct result of the finding that the monohydroxylated metabolite of tamoxifen (Metabolite B) has a high affinity for the estrogen receptor and potent antiestrogenic actions (84). In this section each of tamoxifen's metabolites will be considered in turn.

Monohydroxytamoxifen

It is well established that the relative binding affinity (RBA) of monohydroxytamoxifen for the estrogen receptor is the same or higher than that of estradiol (81, 84–88). This high affinity is responsible for the high potency of monohydroxytamoxifen (50–100-fold increase compared with tamoxifen) for controlling the proliferation of MCF-7 breast cancer cells (88) and inhibiting their secretion of an estradiol-stimulated 46K protein (89) in vitro. In contrast, monohydroxytamoxifen is sufficiently estrogenic in vivo to stimulate progesterone receptor synthesis in the immature rat uterus (42, 90). In terms of antiestrogenic potency in vivo, monohydroxytamoxifen is only 10 times more potent than tamoxifen in uterine weight tests (84). This unexpected finding probably results from the more rapid clearance rate of monohydroxytamoxifen compared with tamoxifen (34). This property of monohydroxytamoxifen is also illustrated in the DMBA-induced rat mammary carcinoma model. While monohydroxytamoxifen can control the growth of established mammary tumors, an equivalent dosage regimen of tamoxifen and monohydroxytamoxifen (50 μg daily for 4 weeks started 30 days after DMBA administration) does not result in monohydroxytamoxifen being more potent in retarding the appearance of mammary tumors (34). Tamoxifen is more effective because it has a longer biological half-life. Nevertheless, continued treatment with either 3 or 50 μg monohydroxytamoxifen daily, starting 1 month after administration of DMBA, can effectively control the appearance of mammary tumors (34). Therefore, biological half-life, as well as potency in vitro, is an important factor in considering the efficacy of a particular metabolite.

Because of its potential as a fundamental research tool, monohydroxytamoxifen has been produced in radiolabeled form with high specific activity (15 Ci/mmol or 42 Ci/mmol). Direct studies with [^{3}H]monohydroxytamoxifen and [^{3}H]estradiol show comparable binding affinities for the estrogen receptor (91). Other than minor differences in ligand affinity (92), no major differences in the physicochemical properties of [^{3}H]estradiol- or [^{3}H]monohydroxytamoxifen–estrogen receptor complexes have been reported. Similarly, studies with monoclonal antibodies to the estrogen receptor show that there is comparable antibody binding to either [^{3}H]estradiol- or [^{3}H]monohydroxytamoxifen–estrogen receptor complexes from breast tumor cytosols (Tate, DeSombre, Green, Jordan, and Jensen, in preparation). Therefore, if the receptor complexes for estrogens and antiestrogens are similar, then the actual shape of the binding ligand may be of critical importance for antiestrogenic action. However, an antiestrogen binding component distinct from the estrogen receptor has recently been reported in human breast carcinoma cytosols (93, 94). This antiestrogen binding component apparently binds [^{3}H]antiestrogens but is not competable by estrogens. The significance of these observations is not known, but these findings suggest that the antiestrogenic (and antitumor) actions of tamoxifen might occur by mechanisms other than via the estrogen receptor.

A major advantage of [^{3}H]monohydroxytamoxifen is that it can be used for the study of antiestrogen binding by tissues in vivo (95, 96). [^{3}H]monohydroxytamoxifen binding in immature rat uterus and vagina is estrogen specific, and prebinding of the radiolabeled ligand in estrogen target tissues is readily reversible by either estradiol or antiestrogens. It is important to appreciate that [^{3}H]monohydroxytamoxifen binds to tissues that

are not estrogen target tissues, e.g., skeletal muscle, spleen, heart, but unlike the situation in target tissues, the ligand is not retained for a prolonged period and there is no competitive interaction with estrogens (96).

Dihydroxytamoxifen

Dihydroxytamoxifen has not been detected in the biological fluids of patients: nevertheless, it has some interesting pharmacological properties. Its RBA for the estrogen receptor is similar to that observed for estradiol (84). Dihydroxytamoxifen is an antiestrogen in the immature rat but exhibits no intrinsic estrogenic activity (84). Furthermore, dihydroxytamoxifen is a partial agonist with antiestrogenic properties in the 3-day ovariectomized mouse uterine weight test (85), though both tamoxifen and monohydroxytamoxifen are full estrogen agonists in the mouse (85).

N-desmethyltamoxifen

Although N-desmethyltamoxifen is the major metabolite of tamoxifen, there are few reports that document its pharmacology. Overall the biological properties seem to be similar to tamoxifen's: the relative binding affinity for the estrogen receptor (97), antifertility activity (97), and antiestrogenic properties in the rat (V.C. Jordan, unpublished observation) are comparable in potency to tamoxifen. Recently N-desmethyltamoxifen has been shown to have a similar potency to tamoxifen's for inhibiting the growth of MCF-7 breast cancer cells (88).

Metabolite E and Metabolite Y

Metabolite E has only been observed in dog bile (69), while Metabolite Y is present in serum of patients on normal or high dose tamoxifen therapy. A comparison of the relative ability of the known metabolites of tamoxifen to inhibit the binding of [^{3}H]estradiol to rat uterine estrogen receptor is illustrated in Fig. 10. Neither Metabolite E nor Metabolite Y is more active than tamoxifen. Metabolite Y is a weak antiestrogen with partial estrogenic activity in the rat uterus. In contrast, Metabolite E is a weak estrogen in immature rat uterine weight tests (98). This pharmacological action is consistent with the literature on triphenylethylenes without a correctly substituted alkylaminoethoxy-side chain (99–103). Recently Metabolite E was reported to inhibit the growth of MCF-7 breast cancer cells (86). In contrast, we have found that Metabolite E can reverse the inhibitory

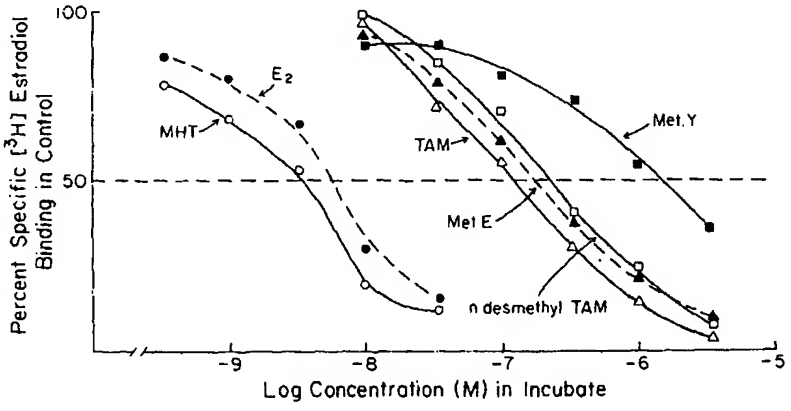


Fig. 10. Inhibition of [^{3}H]estradiol binding to rat uterine estrogen receptors by tamoxifen and its metabolites. Increasing concentrations of monohydroxytamoxifen (○, estradiol (●), as a comparative standard), tamoxifen (△), Metabolite E (▲), N-desmethyltamoxifen (□), and Metabolite Y (■) were incubated with rat uterine cytosol and [^{3}H]estradiol (5 n*M*) for 18 hr at 4°C. Specific binding of [^{3}H]estradiol was calculated from incubates with and without diethylstilbestrol (1 μM), and results were plotted as a percent of specific [^{3}H]estradiol binding.

effects of tamoxifen on MCF-7 cell growth ('estrogen rescue') (Pruitt and Jordan, unpublished observations).

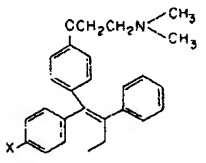
Pharmacological significance of metabolism

The development of assays to measure the relative levels of tamoxifen and metabolites in serum has provided much useful information about the pharmacokinetics of this drug. However, it is worth pointing out that this information, as yet, has not been used to schedule the drug in clinical protocols for breast cancer therapy. This is primarily because tamoxifen has such an advantageous therapeutic ratio and the recommended doses have been derived empirically from early clinical trials.

It is important to identify all the metabolites of tamoxifen in plasma or serum (and determine their pharmacology) because this is a potential reservoir of biologically active ligands. Nevertheless, the relative concentrations of parent compound and metabolites in serum might not reflect the ratio of binding ligands in the tumor or other estrogen target tissue. A recent study (79) documents the plasma levels of tamoxifen and metabolites and also their concentration in tumor tissue. The ratio of tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen (when detection was possible), was similar in plasma and tumor at a concentration that was capable of inhibiting the binding of estradiol to available estrogen receptors. Nevertheless, the relative importance of each binding ligand at equilibrium with the estrogen receptor is unknown. Indeed, the actual binding of ligands to the tumor estrogen receptor in vivo is not established.

The clinical results obtained during continuous antiestrogen therapy contrast with the results observed in rat target tissues after a single administration of [^{3}H]antiestrogen. Studies with several radiolabelled antiestrogens have demonstrated that polar metabolites are preferentially bound in the rat uterus after a single subcutaneous administration (20, 104, 105). Only low levels of [^{3}H]tamoxifen are observed in the rat uterus, but the metabolite monohydroxytamoxifen is observed to accumulate within the cell nucleus along with Metabolite X, an as yet unidentified metabolite (105). Overall these results suggest that antiestrogens are pro-drugs that require metabolic activation to derivatives with a high affinity for the estrogen receptor. However, the pharmacokinetics observed after a single administration are not comparable to the situation during continuous therapy, when high concentrations of parent compound are available to interact with the estrogen receptor. The question of whether the parent compound has biological activity as an antiestrogen must therefore be addressed.

The direct inhibitory effects of tamoxifen on the growth of MCF-7 breast cancer cells are well established (55–57, 88, 89) under conditions where no conversion of tamoxifen to monohydroxytamoxifen can be detected (70, 89). To confirm the generality of these observations both in vivo and in vitro, we recently synthesized a series of substituted derivatives of tamoxifen (Fig. 11) that are unlikely to be metabolized to monohydroxytamoxifen. While introduction of a phenolic hydroxyl into tamoxifen to form monohydroxytamoxifen markedly increases affinity for the receptor, introduction of methyl, chloro, or fluoro essentially does not change the binding affinity of tamoxifen (Fig. 12). To translate these observations into a result with biological relevance, we have determined the direct antiestrogenic properties of these compounds in an estrogen sensitive cell system. We selected the regulation of estradiol-stimulated prolactin synthesis by rat pituitary cells in primary culture. The model system is validated and the assay of prolactin synthesis is precise (106). We have shown (107) that



	X
Tamoxifen	H
Monohydroxytamoxifen	OH
p-methyl Tamoxifen	CH_3
p-fluoro Tamoxifen	F
p-chloro Tamoxifen	Cl

Fig. 11. Structure of tamoxifen derivatives designed to prevent metabolism to monohydroxytamoxifen.

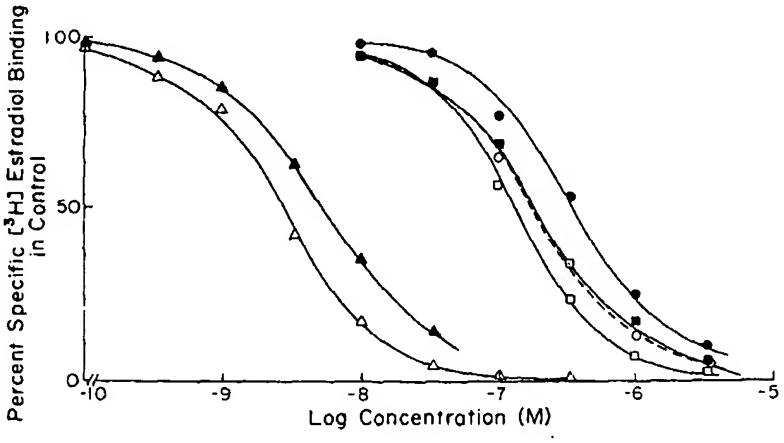


Fig. 12. Inhibition of [^{3}H]estradiol binding to rat uterine estrogen receptors by tamoxifen and its substituted derivatives. Increasing concentrations of monohydroxytamoxifen (△), estradiol (▲, as a comparative standard), *p*-methyltamoxifen (□), tamoxifen (■), *p*-chlorotamoxifen (○), and *p*-fluorotamoxifen (●) were incubated with rat uterine cytosol and [^{3}H]estradiol (5 n*M*) for 18 hr at 4° C. Specific binding of [^{3}H]estradiol was calculated from incubates with and without diethylstilbestrol (1 μM), and results were plotted as a percent of specific [^{3}H]estradiol binding.

the derivatives of tamoxifen inhibit estradiol-stimulated prolactin synthesis at the same concentrations as tamoxifen itself. The antiestrogenic action is competitive with estradiol and reversible with increasing estradiol concentrations. Overall, the potency of each derivative of tamoxifen is related to its RBA for the estrogen receptor. These same derivatives of tamoxifen have been compared with tamoxifen in the three day immature rat uterine weight test to determine their relative partial estrogenic and antiestrogenic potency. Overall, tamoxifen is more potent than its methyl, chloro, and fluoro derivatives, but is not as potent as monohydroxytamoxifen (108). The fact that tamoxifen and its derivatives are equiactive in systems in vitro while tamoxifen is more active in vivo than its derivatives, suggests that fractional conversion of tamoxifen to monohydroxytamoxifen occurs in vivo. It is therefore an advantage for tamoxifen to be converted to monohydroxytamoxifen, but this is not a *requirement* for antiestrogenic activity. If the local concentration of tamoxifen at an estrogen target site is advantageous, for example in a patient's breast tumor during continuous tamoxifen therapy, then an effective antiestrogenic action can occur with the parent drug.

Future considerations

The past few years have seen significant advances in the understanding of tamoxifen's pharmacokinetics and metabolism. However, our knowledge is still incomplete with regard to the identification and pharmacological role of individual metabolites in target tissues and tumors. For the future, there are three broad areas that require investigation:

(1) The species differences of tamoxifen are perplexing. The complete antiestrogenic action of tamoxifen in the chick oviduct compared with the full estrogenic action in the mouse could be viewed from two perspectives. It is possible that the control of estrogen action through a nuclear estrogen receptor-mediated mechanism has varying degrees of species specificity. For example, the nuclear tamoxifen–estrogen receptor complex might be permissive in the mouse because there are less precise structural requirements for the ligand–receptor complex. The structural requirements of the ligand to initiate events in the nucleus of a chick oviduct might be very precise. Alternatively, the control mechanisms of estrogen action are similar for all species, and it is more reasonable to consider that the estrogen receptor is presented with different spectra of binding ligands in different species. The metabolic

handling of tamoxifen in a variety of species should be investigated.

(2) Many of tamoxifen's metabolites have been identified in patients, but none of these are full estrogens that might explain the estrogenic actions. Since most of the estrogenic actions appear to be targeted upon liver functions, it is possible that estrogenic metabolites of tamoxifen are formed to produce a local effect in liver cells, but that the metabolites are rapidly conjugated and excreted via the bile duct. In this way, the estrogens might never get into the systemic circulation at a sufficient concentration to permit detection. The estrogenic effect of tamoxifen in the vagina would seem to be paradoxical unless there are specific mechanisms to scavenge estrogenic metabolites, or an inherent metabolic capacity within the vaginal cells. Further studies of tamoxifen metabolism in patients is essential, with an emphasis on the identification of ligands in target tissues.

(3) Drug interactions have not been studied at all. The impact of tamoxifen on the metabolism and pharmacokinetics of other cancer chemotherapeutic agents should be clarified. Furthermore, the impact of a whole range of other common drugs on the main metabolic routes of tamoxifen should be carefully documented.

The past decade has seen the successful introduction of a new approach to the treatment of breast cancer. Nevertheless, much is still to be resolved concerning the metabolism and subcellular mechanism of action of tamoxifen. For the future, the introduction of new or novel antiestrogens, with different biological properties and different routes of metabolism, will do much to further an understanding of the pharmacology of these drugs.

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Identification of 4-Hydroxy-*N*-desmethyltamoxifen as a Metabolite of Tamoxifen in Human Bile[1]

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ABSTRACT

The occurrence of tamoxifen metabolites in bile was investigated in a 57-year-old female patient receiving chronic treatment with tamoxifen. In bile treated with β-glucuronidase, two major peaks were detected using a chromatographic system developed for the quantitation of tamoxifen metabolites in human serum. One sharp peak coeluted with 4-hydroxytamoxifen whereas a second broad peak eluted slightly ahead of tamoxifen and was separated from all major serum metabolites. This latter peak was identified as the *cis* (about 30%) and *trans* (about 70%) isomers of 4-hydroxy-*N*-desmethyltamoxifen. The identification was based on (*a*) coelution with authentic standard on reversed-phase chromatography and formation of fluorescent material after photoactivation, (*b*) a molecular ion $(M + 1)^+$ of 374 m/z as determined with liquid chromatography-mass spectrometry, and (*c*) a fragmentogram identical to that of the authentic standard, as obtained by gas chromatography-mass spectrometry.

INTRODUCTION

Tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene], is a nonsteroidal antiestrogen, which has been widely used during the last 15 years for the palliative treatment of breast cancer (1).

Metabolism is a major determinant of drug action. Biotransformation may lead to inactivation and excretion, and some metabolites may have pharmacological effects. This is certainly the case for tamoxifen, which may be regarded as a prodrug, since it is converted into more active metabolites (2).

Several metabolites of tamoxifen have been identified in human plasma. These include *N*-desmethyltamoxifen (metabolite X), 4-hydroxytamoxifen (metabolite B), N-desdimethyltamoxifen (metabolite Z), and the primary alcohol named metabolite Y[3] (3). Among the serum metabolites, 4-hydroxytamoxifen has received particular attention since it has higher *in vitro* affinity towards the estrogen receptor than the parent drug (4). The effect *in vivo* after peroral administration is probably limited by high metabolite clearance resulting in low serum concentration (2). However, 4-hydroxytamoxifen remains unmetabolized *in situ* in human breast for several days after local percutaneous administration to human breast (5).

The routes of excretion of [^{14}C]tamoxifen have been investigated in the female rat, mouse, monkey, and dog after i.p. injection or oral administration (6). In all species the major route of excretion was via the faeces. In rats and dogs, a major fraction (22–53%) of radioactivity derived from tamoxifen was excreted into the bile, and a significant amount (16–69%) of biliary radioactivity was reabsorbed and underwent enterohepatic circulation, until it was eliminated into the faeces (6). Investigation of the distribution of [^{14}C]tamoxifen in the female mouse showed that the radioactivity was concentrated and retained in bile and to a lesser degree in liver and pancreas (7).

The excretion of radioactivity following a single oral dose of [^{14}C]tamoxifen has been evaluated by Fromson *et al.* in two female patients. Excretion of metabolites into faeces seems to be a major route of elimination also in humans (8).

Most metabolites isolated from faecal extracts of rat and mouse and bile from rat and dog were present as glucuronides and other conjugates. Three metabolites (metabolite A, B, and C) were identified by comparison with authentic standards, whereas the structural assignment of three metabolites (named C, D, and F) was tentative (6). Fromson *et al.* (8) isolated two metabolites (B and F) from β-glucuronidase treated extract of human faeces. Recently, metabolite F has been identified as the phenolic primary alcohol (9).

The data cited above suggest that the major route of tamoxifen metabolite clearance occurs via biliary excretion. Evaluation of this route of elimination in humans by investigating metabolites in faeces may be obscured by intestinal metabolism and reabsorption of metabolites. However, bile drainage of a patient receiving chronic treatment with tamoxifen offers a clinical condition where biliary excretion of some tamoxifen metabolites could be directly assessed. We obtained such bile and analyzed it with a liquid chromatographic system which we have recently developed for measurement of tamoxifen and its metabolites in human serum (10), This method has the advantage of total analytical recovery of both demethylated and hydroxylated metabolites and the parent compound, and analyzes and quantitates these diverse compounds in a single system. We identified a major peak in human bile as 4-hydroxy-*N*-desmethyltamoxifen.

MATERIALS AND METHODS

Chemicals. Tamoxifen, 4-hydroxytamoxifen and *N*-desmethyltamoxifen, were obtained from Pharmachemie B. V., Haarlem, Holland. Metabolite Y, *N*-desdimethyltamoxifen and 4-hydroxy-*N*-desmethyltamoxifen were gifts from Imperial Chemical Industries PLC, Pharmaceuticals Division, Macclesfield, Cheshire, UK. The latter reference compound was obtained as a mixture of about 85% *cis* isomer and 15% *trans* isomer. Attempts to enrich the *trans* isomer by boiling in ethanol, were not successful. Glusulase (a preparation of the intestinal juice of the snail, Helix Pomatia, containing β-glucuronidase and sulfatase) was from E. I. DuPont de Nemours & Co. Inc., Wilmington, DE. Normotest, testing for blood clotting factors II, VII, and X, is a product of Nycomed AS, Oslo, Norway. Other reagents were purchased from commercial sources given previously (10).

Patient. The patient was a 57-year-old female who had breast cancer with pleural effusion and axillary and liver metastases. She received chronic treatment with tamoxifen (30 mg daily) since 12/18/86. Due to total occlusion of the common bile duct with a serum bilirubin of 303 μM (normal < 18 μM) a T tube biliary drainage was established on 2/6/87. From this date, her liver function parameters improved (Table 1).

On 3/3/87 she received 10 mg Adriamycin, and on 3/5/87 the endocrine treatment was changed from tamoxifen to megestrolacate, 160 mg daily. She was transferred to a local hospital on 3/12/87, and expired on 3/26/87.

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[3] The abbreviations and trivial name used are: metabolite Y, *trans*-1(4-β-hydroxy-ethoxyphenyl)-1,2-diphenylbut-1-ene; LC/MS, liquid chromatography/mass spectrometry; GC/MS, gas chromatography/mass spectrometry.

Table 1 *Patient data*

Date	Serum parameters						
	Bilirubin (μM)	AP[a] (IU/liter)	ASAT[b] (IU/liter)	ALAT[c] (IU/liter)	γ-GT[d] (IU/liter)	Albumin (g/liter)	NT(%)[e]
2/2/87	303	999	117	193	469	23	0.77
2/27/87	75	287		103	186	32	0.65
3/9/87	43	184	51	104	121	29	0.65

[a] Alcaline phosphatase.
[b] Aspartate aminotransferase.
[c] Alanine aminotransferase.
[d] γ-Glutamyl transpeptidase.
[e] Normotest (normal 75–125).

Bile was collected for 5 days (3/1/87–3/5/87) while she received chronic treatment with tamoxifen, and for 20 days (3/5/87–3/25/87) during drug washout. The laboratory findings during this period are summarized in Table 1.

Sample Collection and Processing. The bile was collected in polyethylene bags which were replaced twice daily. The collected bile was stored in glass bottles at −20°C.

The bile was thawed, and treated with glusulase, as described by Bakke *et al.* (11). The pH was then adjusted to 7 by adding 1 M NaOH. The neutralized samples were mixed with equal volume of acetonitrile, and the precipitate removed by centrifugation. The supernates were transferred to sample vials, capped, and analyzed.

High-Performance Liquid Chromatography. We used a liquid chromatography system which was developed for the determination of tamoxifen and metabolites in serum (10). The assay was modified to improve the separation and isolation of the early eluting, hydrophilic metabolites. The method and the modification are briefly described below.

Large samples of 250 μl were injected into a small precolumn with an internal diameter of 0.21 cm, packed with 5 μm octadecylsilane material. The length of this column was increased from 2 cm, as in the original method (10), to 3 cm. The samples were on-column concentrated by equilibrating the precolumn with 50% acetonitrile in water, containing 3 mM acetic acid and 2 mM diethylamine. The analytes were then directed into an analytical octadecylsilane-Hypersil column (0.21 x 10 cm) by changing the mobile phase followed by column switching. The composition of the mobile phase was 91% acetonitrile containing 1 mM acetic acid and 0.67 mM diethylamine, and the flow rate was 0.3 ml/min. Tamoxifen and its metabolites were eluted in the following order: metabolite Y, metabolite B, tamoxifen, metabolite Z, and metabolite X. These compounds were postcolumn converted to fluorophors by UV illumination while passing through a quartz tube, and then monitored by a fluorescence detector.

The instruments and the construction of the postcolumn converter have been described (10).

Liquid Chromatography/Mass Spectrometry. The analytical column was connected to a LC/MS, thermospray system (model 201; Vestec, Houston, TX). Before entering the thermospray, the effluent from the column was mixed with 0.1 M ammonium acetate reagent, delivered at a rate of 0.7 ml/min via a zero dead volume T connector.

Gas Chromatography/Mass Spectrometry. Samples of bile were chromatographed on the liquid chromatographic system described above, and the material to be identified was isolated by collecting effluent from the column, using the programmable fraction collector model, Foxy, from ISCO. The desired fraction from 30 samples were pooled, and lyophilized. The lyophilized sample was silylated with N_iO-bis(trimethylsilyl) acetamid (1 ml), prior to GC/MS analysis.

Samples of 5 μl of the material in ethyl acetate were subjected to GC/MS, using a Hewlett-Packard 5970 MSD (GC/MS) mass spectrometry system. The column was an SP-255 wall-coated, open-tubular glass capillary column (0.30 mm i.d. x 6 m) and the temperature was programmed from 150 to 280°C at 10°C/min. On column injection technique was used, and helium (2 ml/min) was used as a carrier gas.

RESULTS

Tamoxifen and its Metabolites in Serum. Table 2 shows the amount of tamoxifen and its major metabolites in serum at different time points during the investigation. The concentrations of tamoxifen, metabolite Y, *N*-desmethyltamoxifen, and *N*-didesmethyltamoxifen (Table 2) are in the range reported for patients receiving chronic tamoxifen treatment (3, 12). The concentrations of drug and these metabolites in serum are progressively decreased following tamoxifen withdrawal.

Liquid Chromatographic Analysis of Bile. Fig. 1 shows fluorescence traces of UV illuminated effluent from the reversed-phase column.

Chromatogram of bile from a patient not treated with tamoxifen is shown in trace A. No peaks cochromatographed with standards (*traces B* and *C*).

Analysis of bile from a patient receiving chronic treatment with tamoxifen is shown in *traces D* and *E*. Only trace amounts of fluorescent material, tentatively identified as tamoxifen, *N*-desdimethyltamoxifen, and *N*-desmethyltamoxifen, was found in bile not treated with glusulase (*trace D*). Two major peaks appeared following glusulase treatment. One cochromatographed with 4-hydroxytamoxifen and another (retention time of 9 min) eluted slightly ahead of tamoxifen and was separated from all major serum metabolites (shaded area, *traces E* and F). The latter peak was broad and skewed, suggesting inhomogeneity. Our further efforts were directed towards the identification of this peak.

Trace C shows a chromatogram from bile spiked with 4-hydroxy-*N*-desmethyltamoxifen. The standard contained a mixture of *trans* (15%) and *cis* isomer (85%), which showed retention times of 8.95 and 9.42 min, respectively. The minor portion (*trans* isomer) coeluted with the main part of the unknown peak (*trace E*), whereas the *cis* isomer eluted in the down-slope region of the unknown peak. These findings are consistent with the possibility that the unknown material contains a mixture of *trans*- and *cis*-4-hydroxy-*N*-desmethyltamoxifen, and the former isomer is prevailing.

Chromatogram of bile 18 days after the last tamoxifen dose is shown in *trace F*. The material in bile, tentatively identified as 4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen, is markedly decreased during drug washout (Fig. 1, *trace F*).

Identification of Tamoxifen Metabolite in Bile by Mass Spectrometry. Extracted bile and reference compounds were chromatographed on the reversed-phase system described above, and the effluent directed into the mass spectrometry thermospray system.

The spectrum of authentic 4-hydroxy-*N*-desmethyltamoxifen showed only one major peak corresponding to the $(M+1)^+$ ion of 374 *m/z* (data not shown).

Fig. 2 shows a single ion monitoring trace of bile from nontreated patients (*trace A*), bile spiked with 4-hydroxy-*N*-desmethyltamoxifen (*trace B*) and glusulase treated bile from patient receiving chronic treatment with tamoxifen (*trace C*). The instrument was set to monitor 374 *m/z* (4-hydroxy-*N*-desmethyltamoxifen). Material containing the same $(M+1)^+$

Table 2 *Concentrations of tamoxifen and metabolites in serum*

Last tamoxifen tablet given in the morning of 3/5/87. The patient expired on 3/26/87.

Date	Tam[a] (ng/ml)	Met Y[b] (ng/ml)	4-OH-T[c] (ng/ml)	Desdim-T[d] (ng/ml)	Desm-T[e] (ng/ml)
Before tamoxifen withdrawal					
2/27/87	128.3	12.7	3.6	34.4	189.6
3/4/87	153.8	15.2	4.9	32.6	186.6
After tamoxifen withdrawal					
3/9/87	90.1	14.7	4.7	33.3	169.2
3/12/87	61.9	10.1	3.3	30.5	130.5
3/23/87	28.0	4.0	1.8	17.9	67.3

[a] Tamoxifen.
[b] Metabolite Y.
[c] 4-Hydroxytamoxifen.
[d] *N*-Desdimethyltamoxifen.
[e] *N*-Desmethyltamoxifen.

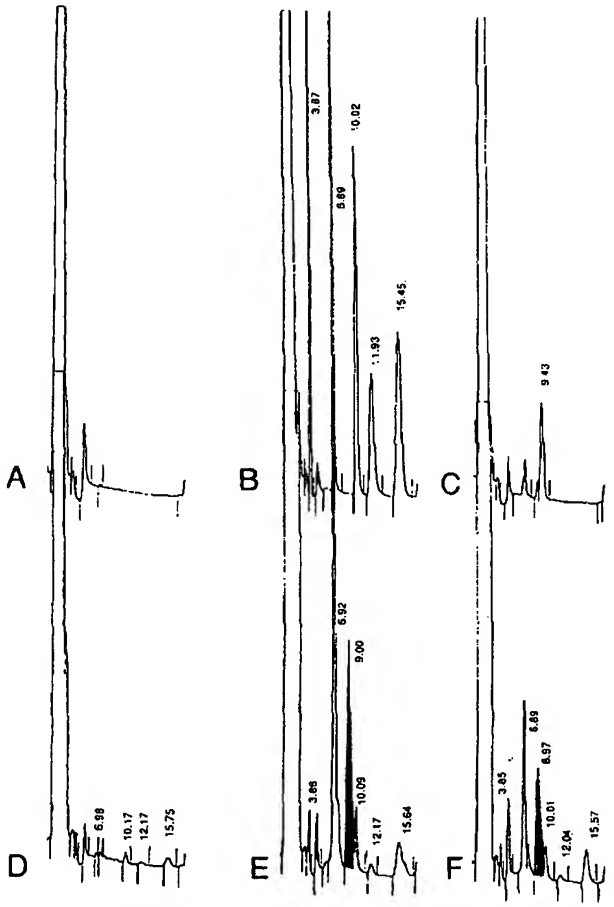

Fig. 1. Analysis of bile by reversed-phase liquid chromatography postcolumn photoactivation and fluorescence detection. *A*, control bile from patient not taking tamoxifen; *B*, blank bile spiked with metabolite Y (3.87 min), 4-hydroxytamoxifen (6.89 min), tamoxifen (10.02 min), *N*-desdimethyltamoxifen (11.93 min), and *N*-desmethyltamoxifen (15.45 min); *C*, blank bile spiked with 4-hydroxy-*N*-desmethyltamoxifen (the standard contained 85% *cis* form and 15% *trans* form); *D*, bile from the patient with biliary drainage, receiving chronic tamoxifen treatment; *E*, the same sample of bile as shown in *trace D*, but after deconjugation with glusulase; *F*, glusulase treated bile from patient 18 days after the last tamoxifen dose. *Shaded areas*, peaks tentatively identified as 4-hydroxy-*N*-desmethyltamoxifen.

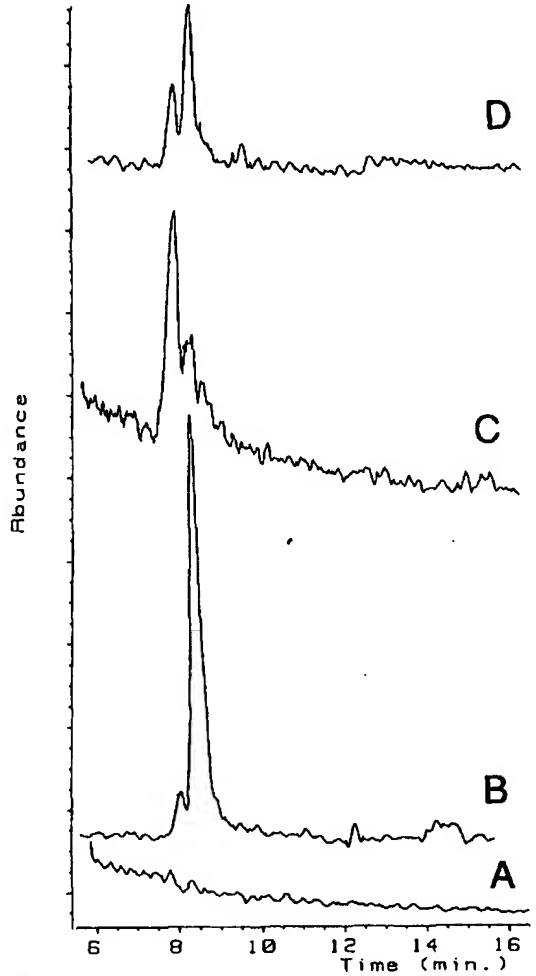

Fig. 2. Single-ion monitoring trace of bile obtained by LC/MS. *A*, control bile from patient not taking tamoxifen; *B*, blank bile spiked with 4-hydroxy-*N*-desmethyltamoxifen (the standard contained 85% *cis* form and 15% *trans* form); *C*, glusulase treated bile from patient receiving chronic tamoxifen treatment; *D*, the same bile as shown in *trace C*, supplemented with authentic 4-hydroxy-*N*-desmethyltamoxifen (85% *cis* form and 15% *trans* form). The instrument was set to monitor at 374 *m/z*, which corresponds to the $(M+1)^+$ ion of 4-hydroxy-*N*-desmethyltamoxifen.

ion and eluting at the same retention times as this reference compound, was present in bile of patient receiving tamoxifen. The standard is composed of 85% *cis* isomer and 15% *trans* isomer (*trace B*) whereas in bile from patient the compound coeluting with the *trans* isomer is the prevailing species (*trace C*). When the bile shown in *trace C* was spiked with standard, there was a relative increase in the *cis* isomer (*trace D*).

Effluent from the peak eluting ahead of the tamoxifen peak (Fig. 1, *shaded area, trace E*) was collected, and analyzed by GC/MS as described in "Materials and Methods." The mass fragmentogram of this material (retention time, 11.92 min) was nearly identical to that of the authentic 4-hydroxy-*N*-desmethyltamoxifen standard (retention time, 11.94 min) (Fig. 3).

DISCUSSION

We analyzed bile from a patient receiving chronic tamoxifen treatment using a chromatographic system which measures both hydroxylated and desmethylated tamoxifen metabolites (10). Notably, the sample processing, which involved mixing with an equal volume of acetonitrile, gave total recovery of such chemically diverse compounds (10). Thus, this analytical procedure includes both hydrophilic and hydrophobic metabolites of tamoxifen.

In addition to 4-hydroxytamoxifen, another major peak was demonstrated in bile. This material was absent in bile from patients not exposed to tamoxifen (Fig. 1, *trace A*) and decreased following drug withdrawal (Fig. 1, *shaded area*, *traces E* and *F*) suggesting that the material is derived from tamoxifen. It was identified as 4-hydroxy-*N*-desmethyltamoxifen. This metabolite appears to exist as a mixture of *cis* and *trans* isomer in bile. The identification was based on (*a*) coelution with authentic standard on reversed-phase chromatography and formation of fluorescent material after photoactivation (Fig. 1), (*b*) a molecular ion $(M + 1)^+$ of 374 *m/z* as determined with LC/MS (Fig. 2), (*c*) a fragmentogram identical to that of the authentic standard, as obtained by GC/MS (Fig. 3).

In bile 4-hydroxy-*N*-desmethyltamoxifen appears as a mixture of *trans* and *cis* isomer, with the former compound as the prevailing species (Fig. 2). If 4-hydroxy-*N*-desmethyltamoxifen is excreted as the *trans* isomer, one must postulate an isomerization of this metabolite during collection or storage of bile. Others have reported on temperature-dependent isomerization of phenolic nonsteroidal estrogens and antiestrogens both in stock solution and in biological samples (13). We observed no time-dependent conversion to the *cis* isomer upon storage of bile. Thus, it is conceivable that 4-hydroxy-*N*-desmethyltamoxifen may be excreted as a mixture of both isomers.

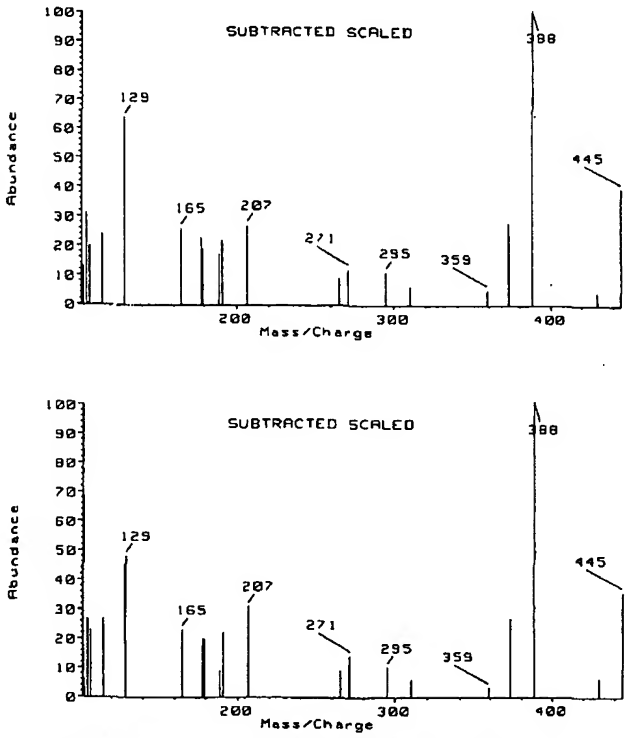

Fig. 3. Identification of the unknown metabolite with GC/MS. The effluent from the reversed-phase column, corresponding to the marked peak in *trace E*, Fig. 1, and authentic 4-hydroxy-*N*-desmethyltamoxifen were subjected to GC/MS as described in the text. Both compounds showed a retention time of 11.9 min upon gas chromatography. *Top*, mass fragmentogram of unknown metabolite; *bottom*, authentic 4-hydroxy-*N*-desmethyltamoxifen.

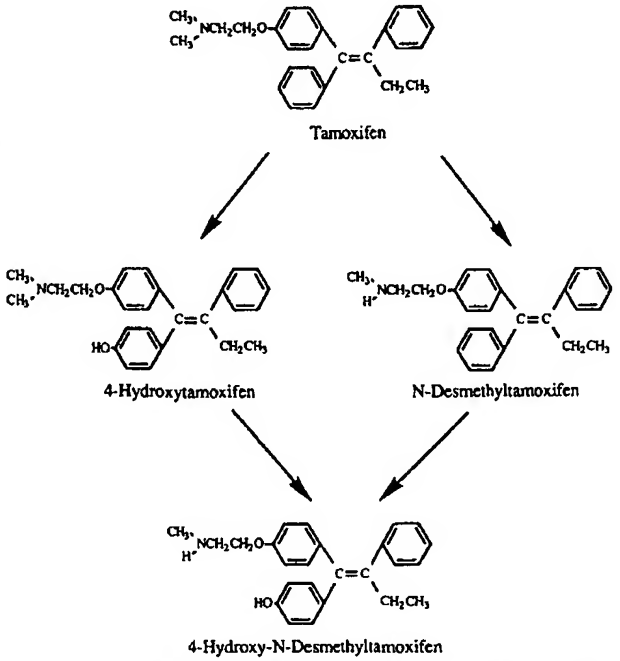

Fig. 4. Possible pathways for the formation of 4-hydroxy-*N*-desmethyltamoxifen.

Since both the *cis* and *trans* form of 4-hydroxy-*N*-desmethyltamoxifen may exist *in vivo* (5), stereoselective metabolism should be considered to take place during biotransformation of tamoxifen. Furthermore, isomerization of active metabolites may have pharmacodynamic implications. It has been established that antiestrogens may change their inhibitory profile after isomerization. The *trans* isomer of tamoxifen is a general estrogen antagonist and the *cis* isomer may act as an agonist (14, 15).

The chromatographic peaks corresponding to 4-hydroxytamoxifen and the two isomers of 4-hydroxy-*N*-desmethyltamoxifen appeared after glusulase treatment of bile, and were nearly absent in nontreated bile (Fig. 1). This finding shows that these hydroxylated metabolites of tamoxifen are excreted and exist as conjugates in bile, probably as glucuronides.

Excretion of conjugated 4-hydroxy-*N*-desmethyltamoxifen and other hydroxylated tamoxifen metabolites into bile delivers these compounds to the intestine, where they are exposed to the intestinal microorganisms. The conjugated metabolites can be degraded by β-glucuronidases or sulfatases derived from bacteria in the gut, and may become reabsorbed and thereby undergo enterohepatic circulation (16). Enterohepatic circulation has been demonstrated for tamoxifen (6) and should be considered for the biliary metabolite, 4-hydroxy-*N*-desmethyltamoxifen.

The existence of 4-hydroxy-*N*-desmethyltamoxifen in human bile points to the possibility that this metabolite is present in tissues as well. The tamoxifen metabolite termed M2 by Borgna and Rochefort, which was found to be a major metabolite in rat uterus (17) may be identical to 4-hydroxy-*N*-desmethyltamoxifen, and significant amount of conjugated 4-hydroxy-*N*-desmethyltamoxifen is formed from tamoxifen by rat hepatocytes in suspension (9). After local percutaneous administration

of 4-hydroxytamoxifen to patients, 4-hydroxy-*N*-desmethyltamoxifen is demonstrated in breast tissue and plasma (5).

There are indications that hydroxylated metabolites of tamoxifen, including 4-hydroxy-*N*-desmethyltamoxifen, play an important role in the mechanism of action of tamoxifen (4). *trans*-4-Hydroxytamoxifen has more than 100 times higher affinity to the estrogen receptor than *trans*-tamoxifen, and a similar *in vitro* affinity has been reported for 4-hydroxy-*N*-desmethyltamoxifen (4).

Possible routes of formation of 4-hydroxy-*N*-desmethyltamoxifen in humans are depicted in Fig. 4. It is conceivable that this metabolite is formed by hydroxylation in the 4-position of *N*-desmethyltamoxifen and/or by *N*-demethylation of 4-hydroxytamoxifen. Both these possible intermediates have been identified in human material (3).

4-Hydroxy-*N*-desmethyltamoxifen has been demonstrated in breast tissue following local administration of the radioactive precursor 4-hydroxytamoxifen. Preliminary data suggest that the concentration of a tamoxifen derivative, tentatively identified as 4-hydroxy-*N*-desmethyltamoxifen, is higher than that of 4-hydroxytamoxifen in serum of several patients receiving chronic p.o. tamoxifen treatment.[4] The unequivocal identification of this serum component by mass spectrometry has been difficult because only small amounts are present in serum. Our present efforts are directed towards improving the detection limit of our LC/MS system so that the distribution of 4-hydroxy-*N*-desmethyltamoxifen in human biological material other than bile can be investigated.

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Decreased Serum Concentrations of Tamoxifen and Its Metabolites Induced by Aminoglutethimide[1]

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ABSTRACT

The antiestrogen tamoxifen and the aromatase inhibitor aminoglutethimide show similar response rates when used in the endocrine management of advanced breast cancer. However, numerous clinical trials have demonstrated no increase in response rate from treatment with the drug combination of tamoxifen plus aminoglutethimide. We investigated the possibility of a pharmacokinetic interaction between these two drugs in six menopausal woman with breast cancer. All patients were investigated under three different conditions (termed phases A, B, and C). The steady state kinetics of tamoxifen were determined when administered alone (phase A) and after coadministration of aminoglutethimide for 6 weeks (phase B). In phase B, the pharmacokinetics for aminoglutethimide were determined and compared with these parameters after a tamoxifen washout of 6 weeks (phase C). The serum concentration of tamoxifen and most of its metabolites {[*trans*-1(4-β-hydroxy-ethoxyphenyl)-1,2-diphenylbut-1-ene], 4-hydroxytamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, *N*-desmethyltamoxifen, and *N*-desdimethyltamoxifen} were markedly reduced following aminoglutethimide administration, corresponding to an increase in tamoxifen clearance from 189–608 ml/min. The amount of most metabolites in serum increased relative to the amount of parent tamoxifen. These data are consistent with induction of tamoxifen metabolism during aminoglutethimide exposure. We found no effect of tamoxifen on aminoglutethimide pharmacokinetics or acetylation. We conclude that this aminoglutethimide-tamoxifen interaction should be taken into account when evaluating the clinical effect of this drug combination relative to monotherapy.

INTRODUCTION

The growth of human breast cancer is supported by endogenous estrogens (1, 2). Tamoxifen and aminoglutethimide are drugs currently used in the endocrine management of breast cancer, and they probably act by suppressing the growth-stimulating effect of estrogens (2–4).

Tamoxifen [*trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene] is a nonsteroidal antiestrogen which is effective against breast cancer in both pre- and postmenopausal women. It is assumed to exert its main effects by blocking the action of estrogens at the receptor site (4). Tamoxifen undergoes extensive hepatic metabolism, and in man metabolites formed by *N*-demethylation are the main circulating species. Significant amounts of hydroxylated metabolites, including the primary alcohol, 4-hydroxytamoxifen, (4) and 4-hydroxy-*N*-desmethyltamoxifen (5) have also been demonstrated in serum. This may be important since some hydroxylated metabolites have higher affinity *in vitro* toward the estrogen receptor than the parent drug, tamoxifen (6–9). Thus, biotransformation of tamoxifen may be an important determinant of drug action. Known metabolites of tamoxifen formed through demethylation and hydroxylation are depicted in Fig. 1.

Aminoglutethimide inhibits the enzyme aromatase, which converts androgens to estrogens in peripheral fat tissue (3). This conversion is the main estrogen source in postmenopausal women. In addition, aminoglutethimide may reduce the concentration of plasma estrogens by enhancement of estrogen metabolism (10, 11). Aminoglutethimide causes response rates in postmenopausal breast cancer patients similar to those of tamoxifen, but because of more frequent side effects aminoglutethimide is generally used after tamoxifen as a second line endocrine treatment (12).

Combination therapy with tamoxifen plus aminoglutethimide should afford both estrogen receptor blockade and reduced plasma estrogen levels, and because of different targeting of these drugs the combination is expected to be more effective than monotherapy. This possibility is supported by studies on human breast carcinoma transplanted into nude mice (13), but the results from clinical trials have been disappointing (14–19) since they all show that the response to tamoxifen is not augmented by adding aminoglutethimide (Table 1).

The reason why the response rate is not increased with combination therapy has not been evaluated. A pharmacokinetic interaction should be considered, especially because aminoglutethimide is a potent inducer of certain hepatic mixed function oxidases and enhances the metabolism of several drugs and steroids (10, 20, 21). In addition, tamoxifen might influence the disposition of aminoglutethimide. Tamoxifen is a potent inhibitor of some mixed function oxidases *in vitro* (22) and may inhibit its own metabolism (23–25) as well as the metabolism of other drugs (26–28).

In the present paper we describe the effect of aminoglutethimide on the disposition of tamoxifen in patients receiving steady state tamoxifen treatment. We also report that tamoxifen does not affect aminoglutethimide disposition. The investigation was motivated by the large number of clinical studies of the combination therapy (Table 1) and also by preliminary findings suggesting that aminoglutethimide alters serum levels of tamoxifen and its metabolites.[3]

MATERIALS AND METHODS

Patients. All patients gave their informed consent to participate in the study. Six postmenopausal women were enrolled. All of them had advanced breast cancer relapsing during tamoxifen therapy and were, therefore, transferred to an aminoglutethimide regimen. Patient characteristics are given in Table 2. All patients had normal liver and renal function tests. One patient (K. N.) did not enter the final part of the study (phase C) because of rapidly progressing disease.

Chemicals. Tamoxifen, metabolite B, and metabolite X were obtained from Pharmachemie B.V. (Haarlem, Holland) and metabolites Y, BX, and Z were gifts from Imperial Chemical Industries, PLC, Pharmaceuticals Division (Macclesfield, United Kingdom). Aminoglutethimide and *N*-acetylaminoglutethimide were gifts from Ciba-Geigy (Basel, Switzerland).

Study Protocol. The study protocol was approved by the regional ethical committee.

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[3] C. Rose and E. A. Lien, unpublished data.

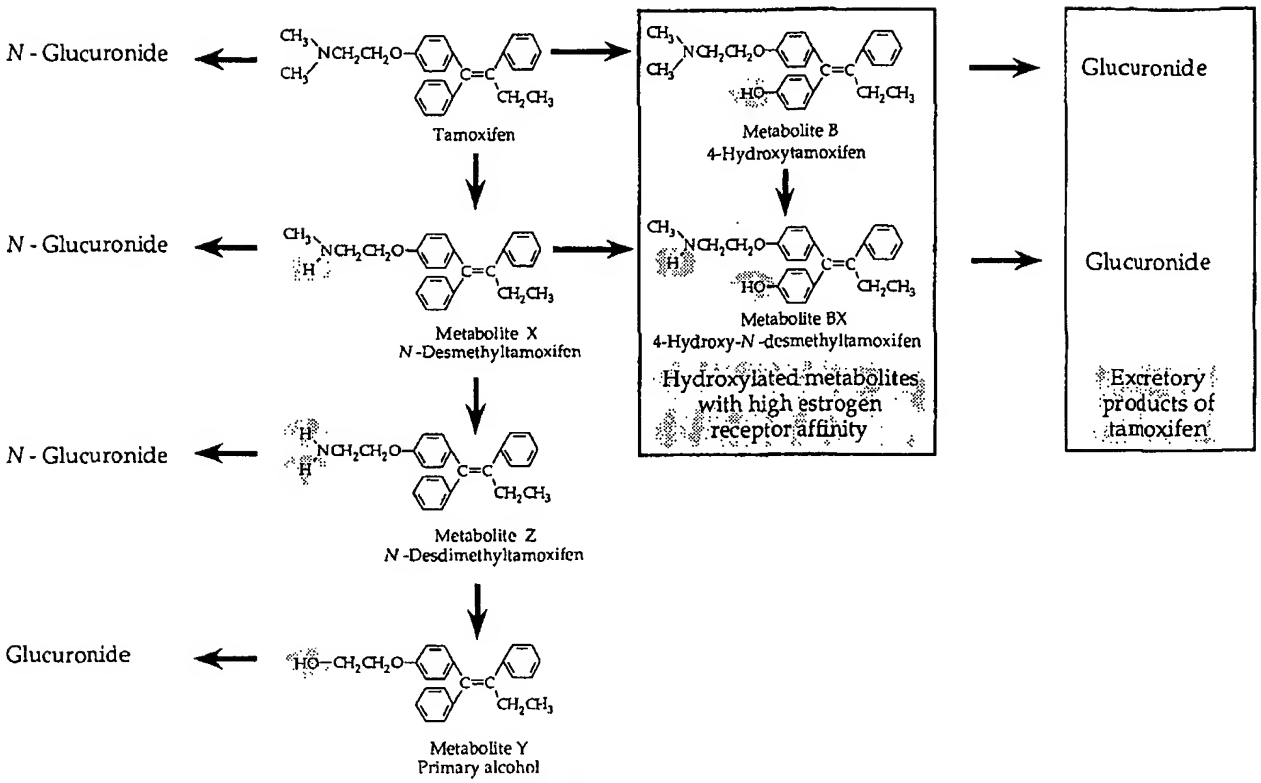

Fig. 1. Proposed metabolic pathways of tamoxifen.

Table 1 *Trials comparing tamoxifen monotherapy with the combination of tamoxifen and aminoglutethimide in breast cancer patients*

Drug[a]	Dose (mg)	Response rate CR + PR *n*[a]	Response rate CR + PR %	Ref.
TAM	10 b.i.d.	18/60	30	14
TAM AG H	10 b.i.d. 250 q.i.d. 20 b.i.d.	23/62	37	
TAM	10 b.i.d.	3/9	33	15
TAM AG H	10 b.i.d. 250 q.i.d. 20 b.i.d.	4/11	36	
TAM	10 b.i.d.	5/26	19	16
TAM AG H	10 b.i.d. 250 q.i.d. 10 b.i.d.	6/26	23	
TAM	10 b.i.d.	21/49	43	17
TAM AG H	10 b.i.d. 250 q.i.d. 10 + 10 + 20	25/51	49	
TAM	10 t.i.d.	32/94	34	18
TAM AG H	10 t.i.d. 250 q.i.d. 20 t.i.d.	24/83	29	
TAM	20 b.i.d.	18/34	53	19
TAM AG H	20 b.i.d. 250 q.i.d. 10 + 10 + 20	11/29	38	

[a] CR, complete response; PR, partial response; *n*, number of patients; TAM, tamoxifen; AG, aminoglutethimide; H, hydrocortisone.

Table 2 *Patient characteristics and drug treatment*

		Treatment		
		Tamoxifen[a]		
Patient	Age (yr)	Duration of treatment before entrance (mo)	Dose (mg)	Aminoglutethimide[b] dose (mg)
A. K.	66	66	30 q.d.	250 q.i.d.
I. L.	60	30	30 q.d.	250 t.i.d.
M. H.	60	41	20 q.d.	250 q.i.d.
B. H.	62	6	30 q.d.	250 q.i.d.
K. N.	47	31	30 t.i.d.	250 q.i.d.
M. F.	60	18	80 q.d.	250 q.i.d.

[a] Phases A and B.
[b] Phases B and C.

Tamoxifen and aminoglutethimide pharmacokinetics were evaluated under three different conditions, termed phases A, B, and C. Drug doses are given in Table 2.

Phase A refers to chronic (>6 months) treatment with tamoxifen given as a single agent. Tamoxifen kinetics and serum levels of its metabolites were determined. For the last 3 days prior to sampling, tamoxifen was given daily at 8 a.m. to all patients after overnight fasting except patient K. N. who received 30 mg t.i.d.[4] at strict 8-h intervals. On the day of investigation, tamoxifen was given at 8 a.m. Blood samples were drawn 0, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 15, and 24 h after the last dose.

Phase B is after treatment with the combination of tamoxifen plus aminoglutethimide and cortisone acetate at fixed doses for 6 weeks. Each patient received the same dose of tamoxifen as during phase A. Aminoglutethimide (250 mg q.i.d) was given with cortisone acetate (50 mg b.i.d for 2 weeks; thereafter 25 mg b.i.d.) as recommended (29). Cortisone acetate is combined with aminoglutethimide treatment because aminoglutethimide blocks the adrenal steroid synthesis (20). During the last 3 days before sampling, tamoxifen was given as in phase A. Aminoglutethimide and cortisone acetate were given at strict 6- and 12-h intervals, respectively. On the day of blood sampling, all drugs were given at 8 a.m. after overnight fasting. Then, cortisone acetate was given after 12 h, and tamoxifen was given after 24 h, but aminoglutethimide was withheld for 48 h. The sampling schedule was as described for phase A with additional samples obtained at 36 and 48 h to allow for determinations of aminoglutethimide half-life.

Phase C is 6 weeks after cessation of tamoxifen therapy. During this period the patients were treated with aminoglutethimide and cortisone acetate only. The kinetics of aminoglutethimide were determined as in phase B.

Blood samples were obtained by venous puncture. Each sample was allowed to clot for 30–60 min prior to centrifugation. Serum was removed and stored at −20°C until analysis. To eliminate between-day variations in the analysis, all samples from each patient were analyzed in the same run.

Determination of Tamoxifen and Its Metabolites. We used a modification of a high performance liquid chromatography assay described previously (30). The method and the modifications are as follows. Samples of 250 μl of serum deproteinized with acetonitrile were post-column on column concentrated on a small precolumn (0.21 x 3 cm), packed with 5 μm ODS material. The analytes were then directed into an analytical ODS Hypersil column (0.21 x 10 cm) by elution and column switching. The mobile phases and other details have been described previously (5, 30). Tamoxifen and its metabolites were post-column converted to fluorophors by UV illumination while passing through a quartz tube and then monitored by fluorescence detection (30).

The within-day precision (CV) of the assay for tamoxifen and its metabolites Y, B, X, and Z were 0.6–5.6% for serum levels between 10 and 800 ng/ml. Because our standard for metabolite BX is a mixture of the *cis* and *trans* isomers (5), the CV was not determined for this metabolite.

Determination of Aminoglutethimide and *N*-Acetylaminoglutethimide. Serum was deproteinized using a mixture of acetonitrile and perchloric acid. The samples were chromatographed on a 3-μm ODS Hypersil column, which was eluted isocratically as described previously (31). The absorbance was routinely recorded at 242 nm.

The CVs for aminoglutethimide and *N*-acetylaminoglutethimide at a concentration of 0.5 μg/ml are 3.9 and 2.6%, respectively.

Identification of Metabolite BX by LC/MS. For patient K. N., all serum samples from phase A and all samples from phase B were pooled in separate tubes. Ten ml from each pool was extracted with 10 volumes of hexane/butanol (98/2, v/v). The supernatant was evaporated in plastic beakers at 55°C under nitrogen, redissolved in 1 ml 50% acetonitrile, and centrifuged. The supernatant was transferred to sample vials, capped, and analyzed. The analytical column was connected to a LC/MS thermospray system (model 201; Vestec, Houston, TX). Before entering the thermospray, the effluent from the column was mixed with 0.1 M ammonium acetate, delivered at a rate of 0.3 ml/min via a zero dead volume T-connector. The flow rate of the HPLC system was 0.7 ml/min.

Pharmacokinetic Calculations. The area under drug concentration-time curve during steady state corresponding to one dose interval was calculated, using the trapezoidal rule (32). Clearance was calculated by the formula:

$$Cl = \frac{F \cdot D}{AUC_\tau^{ss}} \qquad (A)$$

where F is the fraction of the dose (D) absorbed, and AUC_τ^{ss} is the area under the concentration-time curve corresponding to one dosing interval during steady state treatment (33). For aminoglutethimide, F is close to 1 (34). For tamoxifen, the value for F is unknown in humans, but F is close to 1 in animals (35). Because there is indirect evidence of good absorption in man (24), we assumed an F value equal to 1 in all patients under all conditions investigated (phases A and B).

The fraction of drug converted to the metabolite (f_m) is given by the equation (32):

$$f_m = \frac{AUC_{met} \cdot Cl_{met}}{AUC_{drug} \cdot Cl_{drug}} \qquad (B)$$

where AUC_{met} and AUC_{drug} are the area under the serum concentration-time curve for the metabolite and drug, respectively. Cl_{met} is the clearance for metabolite, and Cl_{drug} is the clearance for the parent drug.

Rearrangement of equation B gives:

$$\frac{AUC_{met}}{AUC_{drug}} = f_m \cdot \frac{Cl_{drug}}{Cl_{met}} \qquad (C)$$

A formula expressing the relationship between AUC_{met} and f_m and Cl_{met} was obtained by combining equations A and B:

$$AUC_{met} = f_m \cdot \frac{F \cdot D}{Cl_{met}} \qquad (D)$$

Statistical Methods. The Wilcoxon signed rank test for paired data was used to compare the tamoxifen pharmacokinetic parameters obtained in phases A and B and aminoglutethimide parameters in phases B and C. P values were always expressed as two tailed.

RESULTS

Effect of Aminoglutethimide on Tamoxifen Kinetics and Metabolism. We compared the steady state pharmacokinetics and serum metabolite concentrations of tamoxifen given as a single

[4] The abbreviations used are: t.i.d., 3 times/day; q.i.d., 4 times/day; b.i.d. 2 times/day; q.d., 1 time/day; CV, coefficient of variation; ODS, octadecylsilane; metabolite Y, [*trans*-1(4-β-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene]; metabolite B, 4-hydroxytamoxifen; metabolite BX, 4-hydroxy-*N*-desmethyltamoxifen; metabolite X, *N*-desmethyltamoxifen; metabolite Z, *N*-desdimethyltamoxifen; LC/MS, liquid chromatography/mass spectrometry; HPLC, high performance liquid chromatography; CL, total body clearance; AUC, area under the concentration-time curve; C_{max}, maximum concentration during one dosing interval; C_{min}, minimum concentration during one dosing interval; M, the molecular ion; m/z, the mass to charge ratio.

Table 3 *Effect of aminoglutethimide treatment on tamoxifen pharmacokinetics*

		Tamoxifen			Metabolite Y			Metabolite B			Metabolite BX			Metabolite X			Metabolite Z		
Patient	Aminoglutethimide[a]	C_{max}[b]	C_{min}[c]	AUC[d]	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC	C_{max}	C_{min}	AUC
A. K.	−	113	66	1761	24	13	314	4	3	62	11	6	192	207	141	3804	23	12	370
	+	50	23	767	19	7	271	3	1	41	9	2	117	121	78	2061	18	8	271
	−/+	2.3	2.9	2.3	1.3	1.9	1.2	1.3	3.0	1.5	1.2	3.0	1.6	1.7	1.8	1.8	1.3	1.5	1.4
J. L.	−	160	93	2647	18.1	6	194	10	4	129	8	3	119	264	170	4545	40	20	630
	+	65	24	770	13	4	119	6	2	63	0	0	0	95	64	1909	15	5	238
	−/+	2.5	3.9	3.4	1.4	1.5	1.6	1.7	2.0	2.0				2.8	2.7	2.4	2.7	4.0	2.6
M. H.	−	229	104	2929	8	1	42	18	10	312	55	15	500	308	135	3793	32	12	613
	+	81	36	1052	7	1	53	14	10	248	0	0	0	117	77	2078	13	7	230
	−/+	2.8	2.9	2.8	1.1	1.0	0.8	1.3	1.0	1.3				2.6	1.8	1.8	2.5	1.7	2.7
B. H.	−	433	212	7775	37	16	478	2	0	11	62	33	1166	379	268	7578	62	38	1204
	+	124	44	1494	35	6	315	0	0	0	3	0	2	160	86	2432	33	9	387
	−/+	3.5	4.8	5.2	1.1	2.7	1.5				20.7		583	2.4	3.1	3.1	1.9	4.2	3.1
K. N.[e]	−	356	279	7515	159	112	3208	21	12	409	64	45	1302	1100	860	23277	231	164	4627
	+	93	63	1792	83	54	1531	5	4	94	9	0	150	333	240	6731	100	62	1849
	−/+	3.8	4.4	4.2	1.9	2.1	2.1	4.2	3.0	4.4	7.1		8.7	3.3	3.6	3.5	2.3	2.6	2.5
M. F.	−	323	143	4728	160	61	2171	3	0	35	74	13	770	647	424	12588	109	63	1832
	+	162	31	1558	75	22	845	3	0	39	0	0	0	361	178	5484	78	28	1096
	−/+	2.0	4.6	3.0	2.1	2.8	2.6	1.0		0.9				1.8	2.4	2.3	1.4	2.3	1.7
Mean	−	269	150	4559	68	35	1068	10	5	160	46	19	675	484	333	9264	83	52	1546
Mean	+	96	37	1239	39	16	522	5	3	81	4	0.3	45	198	121	3449	43	20	679
	−/+	2.8	4.1	3.7	1.7	2.2	2.0	2.0	1.7	2.0	11.5	63.3	15.0	2.4	2.8	2.7	1.9	2.6	2.3
Significance[f]	(P) − vs. +			0.032			0.063			0.063			0.032			0.032			0.032

[a] −, without aminoglutethimide treatment; +, during aminoglutethimide treatment; −/+, ratio.
[b] ng/ml.
[c] ng/ml.
[d] ng·h·ml^{-1}.
[e] Patient K. N. used tamoxifen three times daily. *AUC* in this patient is estimated during 8 h and normalized to 24 h.
[f] Wilcoxon signed rank test for paired data.

agent (phase A) with these parameters in the same patients when they were given the drug combination of tamoxifen plus aminoglutethimide for 6 weeks (phase B).

Fig. 2 shows the steady state serum profiles for tamoxifen and its metabolites in patient M. H. during one dosing interval in the absence and presence of aminoglutethimide. In agreement with earlier results (36), the serum levels of tamoxifen and metabolites Y, BX, X, and Z reached a maximum concentration (C_{max}) about 2 h after drug intake (data not shown). The difference between C_{max} and the lowest level during one dosing interval (C_{min}) was reduced for tamoxifen and its metabolites during aminoglutethimide treatment (Fig. 2 and Table 3). Notably, the concentrations of metabolite BX were reduced to near the detection limit during the combination therapy. The results for all 6 patients are summarized in Table 3.

The marked reduction in the amount of metabolite BX in serum during aminoglutethimide treatment (Table 3), was confirmed by mass spectrometry analysis. The LC/MS traces for the $(M + 1)^+$ ion show that this metabolite nearly disappeared in serum during aminoglutethimide treatment (Fig. 3).

Aminoglutethimide caused a significant decrease in *AUC* ($P = 0.032$) for tamoxifen (mean reduction, 73%; range, 80–56%), corresponding to a mean increase in tamoxifen clearance of 222% (Table 4). *AUC* for most metabolites was reduced (mean reduction, about 50%) (Table 3).

The ratio AUC_{met}/AUC_{drug} increased 35–80% during aminoglutethimide treatment for all metabolites, except metabolite BX (Table 5).

Aminoglutethimide Pharmacokinetics and Acetylation. The pharmacokinetics of aminoglutethimide and its metabolite, *N*-acetylaminoglutethimide, were determined in patients receiving chronic treatment with the drug combination of tamoxifen plus aminoglutethimide (phase B) and after tamoxifen was withdrawn for 6 weeks (phase C). In phase C neither tamoxifen nor its metabolites were detected in patient sera, with the exception of metabolite X which was found in low concentrations (<1 ng/ml) in sera from three patients (A. K., M. H., and M. F.). The

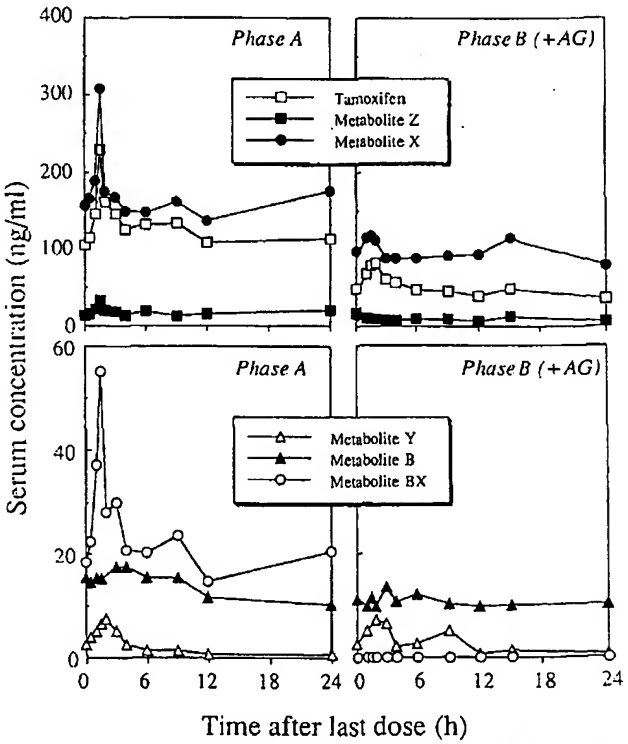

Fig. 2. Serum concentrations curves for tamoxifen and metabolites in patient M. H. during one dosing interval. Phase A is steady state tamoxifen treatment. Phase B is after 6 weeks of combination therapy with tamoxifen and aminoglutethimide. The tamoxifen dose was 30 mg once daily in both phases.

results from a single patient (B. H.) are shown in Fig. 4. Data from all patients are summarized in Tables 4 and 6.

Tamoxifen did not affect the pharmacokinetics of aminoglutethimide or its conversion to *N*-acetylaminoglutethimide (Fig. 4 and Tables 4 and 6).

DISCUSSION

This study demonstrates a pronounced reduction in the serum concentrations of tamoxifen and most of its serum metabolites during aminoglutethimide treatment (Table 3). Several explanations should be considered. Aminoglutethimide may decrease the serum concentration of tamoxifen and its metabolites by reducing the absorption of tamoxifen, reducing tamoxifen protein binding, or by enhancement of tamoxifen metabolism.

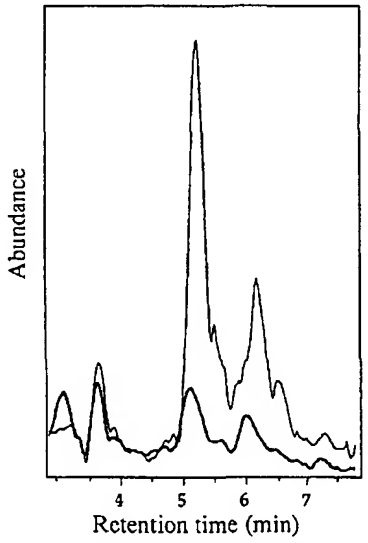

Fig. 3. Chromatography of extracts from pooled sera from phase A and phase B (patient K. N.). Reversed phase LC/MS and sample preparation were performed as described in the text. *Top trace*, selected ion-monitoring trace for the $(M + 1)^+$ ion for metabolite BX (374 m/z) from phase A (tamoxifen as single drug); *bottom trace*, phase B (tamoxifen combined with aminoglutethimide). The second peak eluting after 6 min is due to interference from the tamoxifen peak (372 m/z).

Aminoglutethimide is not known to influence the growth of intestinal bacteria or drug uptake. Thus, there are no data to suggest that aminoglutethimide may impair tamoxifen absorption.

Tamoxifen is highly (>98%) bound by protein in serum (30) and alterations in protein binding may affect the metabolism and distribution of this drug. Because aminoglutethimide is only moderately protein bound (about 25%) (34), it is unlikely that aminoglutethimide can displace tamoxifen from its binding sites.

Our data show that aminoglutethimide reduces the serum level and enhances the elimination of tamoxifen, corresponding to an increase in tamoxifen clearance from 189–608 ml/min (Table 4). This effect from aminoglutethimide is probably due to induction of tamoxifen metabolism, because there is ample evidence that aminoglutethimide may stimulate metabolic processes important in tamoxifen biotransformation.

Tamoxifen is metabolized by hydroxylations and demethylations followed by glucuronidation of the different metabolites as well as of tamoxifen itself (Fig. 1) (4, 35, 37). Aminoglutethimide is an efficient inducer of cytochrome P450 mixed function oxidases (10, 20, 21, 38, 39), and it shows similarities with phenobarbital in this respect (40). Treatment of rats with

Table 4 *Interaction between aminoglutethimide and tamoxifen*

Patient	Clearance of tamoxifen (ml/min)		Clearance of aminoglutethimide (ml/min)	
	−AG[a]	+AG[b]	−TAM[c]	+TAM[d]
A. K.	284	652	105	84
I. L.	189	649	113	87
M. H.	114	317	175	235
B. H.	64	335	111	107
K. N.[e]	200	837		
M. F.	282	856	71	86
Mean	189	608	115	120
Significance (P)[f]	0.032		>0.20	

[a] No aminoglutethimide, phase A.
[b] Aminoglutethimide treatment, phase B.
[c] No tamoxifen, phase C.
[d] Tamoxifen treatment, phase B.
[e] K. N. did not enter the final part of the study because of rapidly progressing disease.
[f] Wilcoxon signed rank test for paired data.

Table 5 *Effect of steady state aminoglutethimide treatment on the amount of tamoxifen metabolites relative to parent drug in serum*

Patient	Aminoglutethimide	AUC^{ss} for metabolite/AUC^{ss} for tamoxifen				
		Y	B	BX	X	Z
A. K.	−	0.18	0.04	0.11	2.16	0.21
	+	0.35	0.05	0.15	2.69	0.35
I. L.	−	0.07	0.05	0.04	1.72	0.24
	+	0.15	0.08	<0.001[b]	2.48	0.31
M. H.	−	0.01	0.11	0.17	1.29	0.21
	+	0.05	0.24	<0.001[b]	1.98	0.22
B. H.	−	0.06	0.001	0.15	0.97	0.15
	+	0.21	0.00	0.001	1.63	0.26
K. N.	−	0.43	0.05	0.17	3.10	0.62
	+	0.85	0.05	0.08	3.76	1.03
M. F.	−	0.46	0.01	0.16	2.66	0.39
	+	0.54	0.03	<0.001[b]	3.52	0.70
Mean	−	0.20	0.04	0.13	1.98	0.30
	+	0.36	0.07	0.04	2.68	0.47
P − vs. +[c]		0.032	>0.10	0.032	0.032	0.032

[a] AUC^{ss}, AUC in steady state during one dosing interval.
[b] BX not detectable during aminoglutethimide therapy.
[c] Wilcoxon signed rank test for paired data.

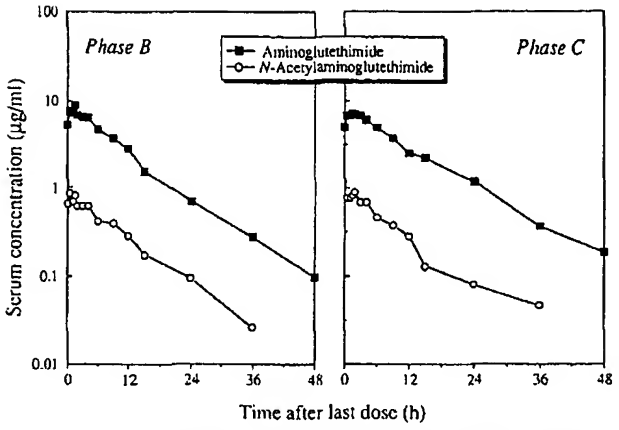

Fig. 4. Serum concentration curves for aminoglutethimide and *N*-acetylaminoglutethimide in patient B. H. Phase B is after 6 weeks of combination therapy with tamoxifen and aminoglutethimide. Phase C is during administration of aminoglutethimide as a single agent 6 weeks after cessation of tamoxifen treatment. In both phases the kinetics of aminoglutethimide were recorded during a period of 48 h of withdrawal of this drug.

barbiturate increases demethylation of tamoxifen in liver microsomes *in vitro* (41).

Induction of glucuronidation has been reported in man after treatment with other well-known enzyme inducers such as phenytoin, phenobarbital, and rifampicin (42), and recently rat liver glucuronidation was found to be enhanced by aminoglutethimide (43). The two hydroxylated metabolites, B and BX, are excreted in bile (5). Their biliary excretion as glucuronides may significantly contribute to their total clearance, and induction of glucuronidation of hydroxylated tamoxifen metabolites by aminoglutethimide may decrease serum levels of these species.

N-Glucuronidation of tertiary amines has been demonstrated only in higher primates (42), suggesting a metabolic pathway for tamoxifen in man, not existing in most experimental animals. Stimulation of tamoxifen *N*-glucuronidation (Fig. 1) by aminoglutethimide would enhance the metabolic clearance of the drug but cannot explain the altered ratio between AUC for a metabolite relative to that of the parent drug.

The observation that AUC for tamoxifen metabolites is reduced (Tables 3 and 5) also agrees with the idea that aminoglutethimide affects tamoxifen metabolism. Our study does not allow delineation of the kinetics behind the reduction in metabolite AUC. According to equation D, AUC_{met} depends on the fraction of tamoxifen converted into the metabolite as well as on the metabolite clearance. These (f_m and Cl_{met}) are parameters not accounted for by the present study design. However, reduction of AUC for tamoxifen metabolites may be due to reduced f_m, or increased Cl_{met}. Reduction in f_m may result if aminoglutethimide stimulates the formation of metabolites not detected by our HPLC system, which was optimized for the analysis of triphenylethylenes present in human serum during monotherapy (30). Increased metabolite clearance may occur following enhancement of metabolic glucuronidation.

Our patients were given cortisone acetate as a glucocorticoid substitution during aminoglutethimide treatment. There is evidence that corticosteroids may affect the metabolism of some drugs (40, 44). We do not consider cortisone acetate responsible for the observed alteration in tamoxifen metabolism for two reasons. First, aminoglutethimide is an inhibitor of adrenal cortisol synthesis, and the cortisone acetate substitution does not increase plasma cortisol above physiological levels (34). Second, aminoglutethimide is an enzyme inducer also in the absence of glucocorticoid substitution (39).

The effect of aminoglutethimide on tamoxifen metabolism has important implications. Obviously, lowering the serum concentration of tamoxifen and its active metabolites reduces their effects. In addition, aminoglutethimide increases the relative amount in serum of most metabolites compared with the parent drug (Table 5). This also suggests that the tamoxifen-aminoglutethimide interaction is due to increased metabolism and not decreased gastrointestinal absorption (see above). An increased ratio AUC_{met}/AUC_{drug} is observed for the hydroxylated metabolite B, whereas the ratio decreases for metabolite BX, another hydroxylated metabolite. These metabolites have considerably higher affinity for the estrogen receptor than tamoxifen itself (6–9). It has recently been demonstrated that the inhibition of growth of the estrogen receptor-positive MCF-7 cells in the presence of tamoxifen and metabolites Y, B, X, and Z parallels the relative affinity of these agents for the estrogen receptor (45). Effects of higher doses of tamoxifen given in combination with aminoglutethimide may therefore be influenced by the altered metabolite profile of tamoxifen.

Tamoxifen is a weak estrogen agonist and strong antagonist, and tamoxifen metabolites may also have agonistic or antagonistic properties (46). Thus, alterations in tamoxifen metabolism induced by aminoglutethimide may increase the amount of estrogen agonists at the expense of estrogen antagonists. Such a metabolic effect would counteract the biological effect of aminoglutethimide thought to be mediated by estrogen depletion, and a decreased additive effect of the drug combination of tamoxifen and aminoglutethimide would ensue. This could explain the negative results from the clinical trials of this drug combination.

Our results also show major variations in ratios of tamoxifen to its metabolites in the absence of aminoglutethimide therapy (Table 3). This raises the question that breast cancer patients who respond to tamoxifen therapy may have a tamoxifen metabolism different from that of the nonresponders.

There are occasional reports that tamoxifen interacts with other drugs (26–28). We observed no effect of tamoxifen ad-

Table 6 *Effect of tamoxifen treatment on aminoglutethimide pharmacokinetics and acetylation*

Tamoxifen[a]		Aminoglutethimide AUC_τ^{ss}[b] (μg·h·ml^{-1})	Aminoglutethimide $T_{\frac{1}{2}}$ (h)	Aminoglutethimide V_Z[c] (liter)	*N*-Acetylaminoglutethimide AUC_τ^{ss} (μg·h·ml^{-1})
+	Mean[d]	40.5	7.4	76.1	4.2
	SD	13.5	1.4	42.1	2.4
−	Mean	39.3	7.2	75.8	3.8
	SD	12.4	1.8	38.7	1.6

[a] +, during tamoxifen treatment (phase B); −, without tamoxifen (phase C).
[b] AUC in steady state during one dosing interval.
[c] Pharmacokinetic volume of distribution during terminal phase.
[d] $n = 5$.

ministration on the disposition of aminoglutethimide (Tables 4 and 6).

In conclusion, the present report demonstrates that aminoglutethimide markedly reduces serum concentrations of tamoxifen and its metabolites, probably by inducing tamoxifen metabolism. Our findings suggest that clinical trials performed on tamoxifen plus aminoglutethimide combined therapy (14–19) may be biased by low tamoxifen serum levels and change in its metabolite profile. This may explain why combination therapy did not result in significantly higher response rates than tamoxifen monotherapy (Table 1). Future clinical trials of the combination therapy should therefore include serum concentration monitoring and tamoxifen doses should possibly be increased to compensate for decreased bioavailability of tamoxifen and its metabolites.

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REVIEW ARTICLE

Clinical Potential of New Antiestrogens

By William J. Gradishar and V. Craig Jordan

Purpose: **Based on the data and clinical experience derived from tamoxifen usage, the properties of an ideal antiestrogen is described that could have applications as a breast cancer preventative agent, long-term adjuvant therapy, or as a treatment for osteoporosis. Each of the new antiestrogens currently being tested is discussed in terms of laboratory development, toxicology, pharmacology, endocrinology, and clinical evaluation. And each new compound is assessed according to the properties of an ideal antiestrogen.**

Methods: **A review of all published reports was facilitated by the use of Medline computer searches.**

Results: **Numerous compounds are being evaluated in clinical trials and can be categorized as triphenylethylenes or tamoxifen analogs, pure antiestrogens, and targeted antiestrogens. Several of these compounds may have fewer uterotropic properties and greater effects on maintaining bone density compared with tamoxifen; however, the clinical experience (ie, patient-years of treatment) with any of these compounds is minimal.**

Conclusion: **Although many of these compounds appear promising, further evaluation will be necessary to determine the role these compounds may serve as preventive agents, adjuvant therapies, treatments for advanced disease, or other medical indications such as osteoporosis.**

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TAMOXIFEN, a nonsteroidal antiestrogen, has been used as a treatment for breast cancer for a quarter of a century.[1-4] It is clear that antiestrogen treatment has revolutionized breast cancer therapy and is established as the endocrine therapy of choice for all stages of the disease.[5] There are currently more than seven million patient-years of clinical experience with tamoxifen, and continuing research has demonstrated additional physiologic advantages and potential disadvantages of the drug based on its target site-specific actions.[6] The differential actions of tamoxifen occur by selective estrogen receptor (ER) modulation at different target sites to produce estrogenic or antiestrogenic effects. For example, long-term tamoxifen therapy produces estrogen-like effects that maintain bone density and lower circulating low-density lipoprotein cholesterol.[7] This latter estrogenic effect appears to translate into a lower incidence of myocardial infarction.[7] In contrast, there is the possibility that tamoxifen eventually produces drug resistance,[8,9] and the fact that tamoxifen produces liver cancer in rats at high doses[10,11] has naturally resulted in concern about the potential to produce second maligancies. Specifically, tamoxifen is associated with an increased detection of endometrial cancer.[12,13] Despite the fact that the International Agency for Research on Cancer has recently evaluated tamoxifen and found evidence of carcinogenicity, the Agency has stated that the World Health Organization considers tamoxifen to be an essential drug for the treatment of breast cancer. No woman should be denied the benefits of tamoxifen because of a concern about the modest risk of developing endometrial cancer (ie, the benefits far outweigh any of the risks). The concerns about the carcinogenicity of tamoxifen have been reviewed recently with regard to the clinical relevance of current data.[11,12,14] At present, there is little clear-cut clinical evidence or description of serious unpredicted side effects to support the hypothetical toxicities. Nevertheless, the success of tamoxifen has proved to be invaluable for the development of new and novel antiestrogens to be used as preventive agents, long-term adjuvant therapies, or as novel treatments for osteoporosis. We will evaluate each available new agent, based on the criteria required for the ideal therapeutic agent, so that clinicians can make an objective assessment of the information that is used to support the claims for each new antiestrogens under investigation.

THE IDEAL AGENTS FOR THERAPEUTIC EVALUATION

There are three distinct goals for drug discovery to exploit the current therapeutic applications of antiestrogens. The opportunities are illustrated in Fig 1. Tamoxifen is being used experimentally as a preventive for women at high risk of breast cancer, and also as a treatment for all stages of breast cancer. One strategy is to develop agents that are not cross-resistant with tamoxifen and have a safer toxicity profile. Another strategy is to develop a pure (ie, nonestrogenic) antiestrogen that does not have stimulatory effects in the uterus and does not cause premature drug resistance. These agents could find use as second-line therapies after the failure of tamoxifen or as optimal estrogen antagonists for the first-line treatment of metastatic breast cancer.

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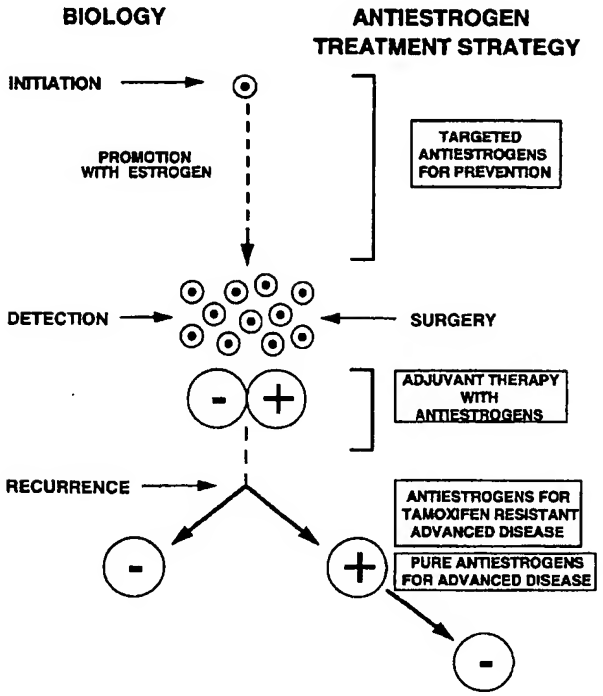


Fig 1. Treatment opportunities with antiestrogens. New antiestrogens could be discovered that do not develop antiestrogen-stimulated tumor growth and are not cross-resistant with tamoxifen. Pure antiestrogens could be used for advanced disease, but targeted antiestrogens could be used as preventives for breast cancer and as specific hormone replacement therapies for postmenopausal women.

Finally, an ideal agent for adjuvant therapy, and as a preventive, could be designed to exploit the beneficial effects of tamoxifen on bones and lipids by solving the problems suggested by an increased detection of endometrial cancer and the theoretical concerns about DNA adduct formation in rat liver. Furthermore, as quality-of-life issues become of primary importance for the application of antiestrogens in women without a diagnosis of breast cancer, a critical area for research consideration will be the control of hot flashes and menopausal symptoms. Clearly, a compound that mimics estrogen in the brain would be a major advantage for patient compliance.

The properties of an ideal agent that could be used more widely, eg, as a hormone replacement therapy in postmenopausal women, are illustrated in Fig 2. If these properties can be achieved, the agents could be applied to a host of novel situations throughout medicine as treatments and preventatives for osteoporosis and coronary heart disease. If a targeted antiestrogen was also antiestrogenic in the breast and uterus, then the new therapy would also prevent breast and endometrial cancer as a beneficial side effect of its primary therapeutic goal. We describe the progress that has been made during the past decade to achieve three separate goals: the development of a safer antiestrogen, a pure antiestrogen, and a targeted antiestrogen.

DISCOVERY OF NEW ANTIESTROGENS

The intense investigation of the pharmacology of tamoxifen during the 1970s and 1980s established a data base for the development of new compounds to treat breast cancer and for broader therapeutic applications. During the 1960s and 1970s, all of the available antiestrogens were noted to have a low affinity for the ER.[15] This observation lead to the widely held belief that antiestrogens had to be compounds with low affinity for the ER so that the receptor complex would break up before the functions required for estrogen action could be completed.[15] The compounds were thought of as weak estrogens that inhibited full estrogen action by saturating all available ER. The discovery that the metabolites of tamoxifen, 4-hydroxytamoxifen and 3,4-dihydroxytamoxifen, have a high affinity for the ER and inhibit full estrogen action, changed the understanding of antiestrogen action.[16] Receptor binding and biologic activity are now viewed as two separate functions in the same molecule.

The principle of an antiestrogen with high affinity for the ER has been exploited with the antiestrogens, TAT-59 and 3-hydroxytamoxifen (droloxifene), that mimic the metabolites of tamoxifen (Fig 3). TAT-59 is a phosphorylated derivative of 4-hydroxytamoxifen that requires dephosphorylation to the active antiestrogen.[17] In contrast, droloxifene is derived from the tamoxifen metabolite 3,4-dihydroxytamoxifen. This metabolite of tamoxifen is unusual because it has antiestrogenic activity in both rat and

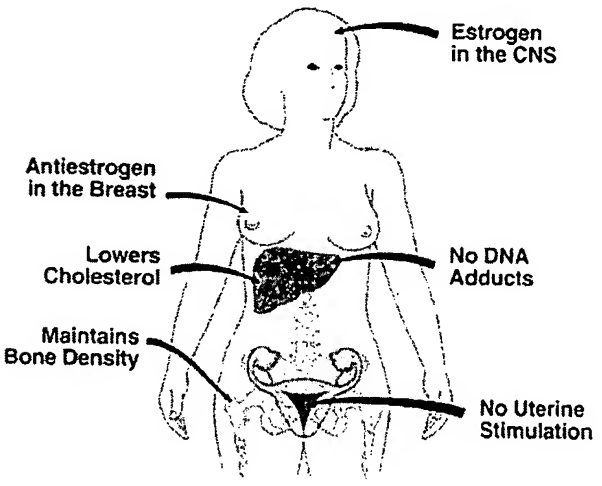


Fig 2. Properties of an ideal targeted antiestrogen to be developed as a new hormone replacement therapy for postmenopausal women.

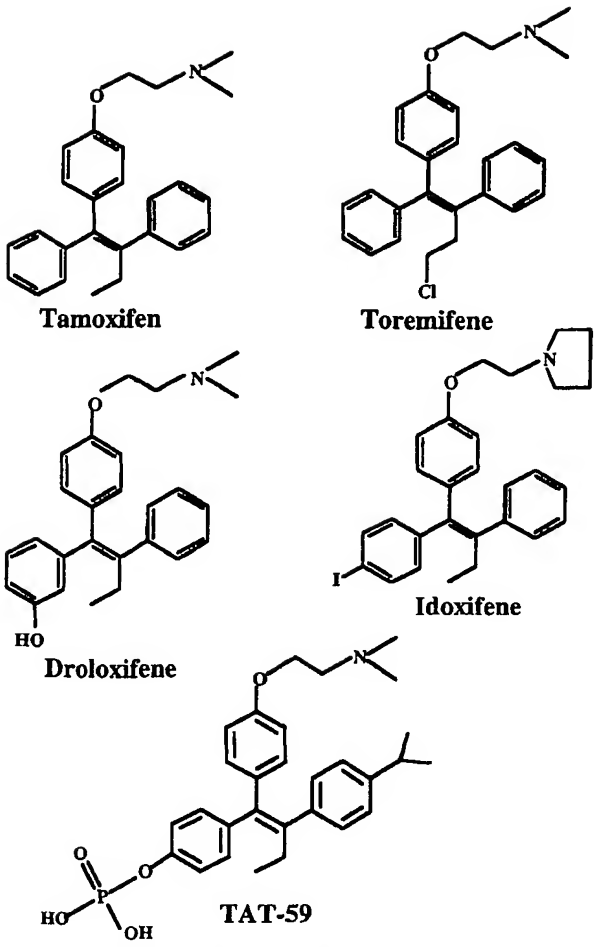


Fig 3. Formula of nonsteroidal antiestrogens related to tamoxifen.

mouse uterine weight tests,[16,18] whereas tamoxifen and 4-hydroxytamoxifen are both classified as an estrogen in short-term mouse assays.[16] 3,4-Dihydroxytamoxifen is very unstable and is readily deactivated by the enzyme catechol orthomethyltransferase; therefore, it would be unpromising as a therapeutic agent.[19] However, removal of the 4-hydroxy to produce droloxifene retains antiestrogenic properties,[20] and most importantly, antitumor properties.

The Eli Lilly Company (Indianapolis, IN) has made a systematic study of compounds that avoid the triphenylethylene structure but retain potent antiestrogenic properties. The antiestrogen trioxifene was discovered[21] during the late 1970s and has some structural similarities to an earlier unsuccessful compound nafoxidine. Trioxifene is active as an antitumor agent in the laboratory[22] and in the treatment of advanced breast cancer,[23-25] but it does not have the severe side effects noted earlier with nafoxidine. Although trioxifene showed some promise, it has not been developed as a clinically useful agent because side effects were believed to be a potential problem when compared with tamoxifen.

In contrast to tamoxifen, the novel antiestrogens LY117018 and LY156758 have a high affinity for the ER and are less estrogenic in the rat and mouse uterus compared with tamoxifen.[26-29] These compounds are also potent inhibitors of estrogen-stimulated effects in vitro.[30-32] Additionally, the novel antiestrogens show antitumor properties in animal models but are not superior to tamoxifen.[33-35] However, and perhaps most importantly, the weakly estrogenic, antiestrogen keoxifene (LY156758) was found to maintain bone density in the ovariectomized rat.[36] The drug has subsequently been renamed raloxifene and has entered clinical trials for the treatment of osteoporosis after confirmation[37,39] of the original animalstudies.[36]

The possibility that a pure antiestrogen could be developed with high binding affinity for the ER combines the observation that MER-25, the first antiestrogen, has virtually no estrogenic properties in any animal species,[40] with the knowledge that binding affinity and biologic activity are separate functions of the same molecule.[16] The antiestrogens ICI 164,384 and ICI 182,780 are derivatives of estradiol with an optimal binding affinity for the ER, but these structural analogs are unique because they do not have any estrogenic properties[41] and they have a novel subcellular mechanism of action that involves the destruction of newly synthesized ER.[42,43] The pure antiestrogen ICI 164,384 has been used extensively in laboratory studies, but the more potent ICI 182,780[44] is being evaluated as a second-line therapy for the treatment of breast cancer after the failure of long-term adjuvant tamoxifen.[45]

Finally, for the past 15 years, there has been a search for tamoxifen derivatives that might have less clinical toxicity. Toremifene, or chlorotamoxifen,[46,47] is less potent than tamoxifen as an antiestrogen[48] and an antitumor agent,[49] which has translated into higher daily doses of toremifene being used in clinical trials to treat advanced breast cancer.[50] The most interesting feature of the toxicology is that the compound does not produce liver tumors in rats.[51,52] Clearly, this property of toremifene could become important if tamoxifen is proven to be a hepatocarcinogen in humans. In contrast to toremifene, idoxifene was designed to be metabolically resistant so that there would be less likelihood of carcinogenic potential. Substitution of a halogen atom at the 4 position of tamoxifen to prevent metabolic activation to 4-hydroxytamoxifen is known to reduce antiestrogenic potency.[53] It is therefore an advantage, but not a requirement, for the antiestrogen tamoxifen to be metabolically activated.[54,55] To exploit this concept, idoxifene was designed with an iodine atom

at the 4 position of tamoxifen to prevent toxicity through 4-hydroxylation and a pyrrolidino side chain to avoid theoretical toxicities associated with demethylation.[56,57] The compound is entering clinical trials, but it is unclear whether idoxifene will be free from carcinogenic potential.

In summary, several new compounds have been discovered that are now actively being evaluated in clinical trials and should be approved for different treatment applications in the next 5 years.

THE EVALUATION OF NEW ANTIESTROGENS

A number of novel compounds are being evaluated in clinical trials, but for convenience, we have divided them into three major categories based on their proposed applications: (1) triphenylethylenes (tamoxifen analogs); (2) pure antiestrogens; and (3) targeted antiestrogens. We will consider each new compound by discussing the laboratory evidence to support the clinical testing currently in progress.

Triphenylethylenes (tamoxifen analogs)

Toremifene

Toremifene (Fig 3), or chlorotamoxifen, began development in Finland by Farmos Pharmaceuticals (Orion, Turku, Finland) in 1978. The drug is approved by the Food and Drug Administration (FDA) for the treatment of metastatic breast cancer and is marketed in the United States under the trade name of Farnesdon R by Scheering Plough (Kenilworth, NJ). The recommended treatment regimen for metastic breast cancer in postmenopausal women is at least 60 mg daily.

Laboratory studies. Toremifene has a biphasic effect on the growth of ER-positive, MCF-7 breast tumor cells in vitro.[46,47] At low concentrations (10^{-7} to 10^{-6} mol/L), toremifene is an estrogen antagonist, but at high concentration ($>10^{-6}$ mol/L), toremifene is oncolytic, and the effects cannot be reversed by estrogen.[57] Nevertheless, toremifene can be washed out of the cells and estradiol can restimulate growth. Toremifene and other antiestrogens increase the production of transforming growth factor-beta (TGF-β),[58] and toremifene-induced cell death has been documented as apoptotic.[59]

Toremifene is an antiestrogen in the immature rat uterine weight test and shows the properties of a partial estrogen antagonist[48] (ie, toremifene causes modest increases in uterine weight when administered alone). Toremifene is effective in controlling the growth of dimethylbenzanthracene (DMBA)-induced rat mammary carcinomas,[48,49] but appears to be approximately one third less potent than tamoxifen.[49] This observatiom is consistent with the larger dose of toremifene (60 mg daily) that is used clinically compared with tamoxifen (20 mg daily).

The MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) breast cancer cell lines will grow in athymic mice. However, toremifene will only block the estrogen-stimulated growth of MCF-7 cells, but does not inhibit the growth of MDA-MB-231 cells.[60] The drug does not control the growth of mixed tumors containing both MDA-MB-231 and MCF-7 cells.[60] Nevertheless, toremifene has been reported to have a cytolytic effect on the growth, in vivo, of an ER-negative, glucocorticoid-sensitive mouse uterine sarcoma.[46] Toremifene can, however, produce aquired resistance after long-term therapy. Athymic animals implanted with MCF-7 cells will eventually grow tumors in response to toremifene treatment.[61]

Toxiclogy. Toremifene does not produce a mutagenic effect in either the Ames test or the sister chromatid exchange assay.[46,62] Large daily doses (5 to 20 mg) of tormifene do not produce DNA adducts in the rat liver, and long-term therapy does not result in hepatocarcinogenesis.[51,52] However, recent studies demonstrate that large doses of toremifene (750 mg toremifene per kilogram of food) can promote rat liver and kidney carcinogenesis.[63]

Clinical pharmacology and endocrinology. Toremifene is extensively metabolized in animals and patients.[64,65] Toremifene can be measured using high performance liquid chromatography (HPLC),[66-69] and the time to steady-state[70] and terminal elimination half-life[64] have been determined as 2 weeks and 5 days, respectively. Toremifene shows weak estrogen-like properties in the postmenopausal patient. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are slightly depressed during therapy, and sex hormone binding globulin (SHBG) is increased.[71-73] Although toremifene shows antiestrogenic effects on the vaginal mucosa of estrogen-primed women,[74,75] there is no effect in blocking short-term estrogen action in the uterus.[75] Toremifene and tamoxifen produce the same estrogen-like effects on the histology of the postmenopausal endometrium.[76]

Clinical evaluation. The initial phase I studies started in the early 1980s demonstrated that toremifene was well tolerated with minimal toxicity, and activity in breast cancer was observed.[66,71,77] Several phase II clinical trials of toremifene have been reported in postmenopausal patients with advanced disease who did not receive prior hormonal or cytotoxic chemotherapy.[78-83] In 46 previously untreated patients with ER-positive metastatic breast cancer, Valavaara et al[78] reported a 63% objective response rate (complete response [CR] 37%; partial response [PR] 26%) after treatment with toremifene (60 mg/d orally). Responses were observed in soft tissue and visceral sites

of disease. No significant differences in response rates could be detected when related to different ER concentrations. Toxicity was mild, with hot flashes occurring in 22% of patients. Gunderson[79] reported a 48% response rate, including six CRs, using an identical treatment schedule of toremifene in a group of 23 patients with advanced disease, 20 of whom had received no prior therapy. The median duration of CRs and PRs was 14 and 15 months, respectively. Hot flashes were reported in approximately half of the patients. Similar findings have been reported by Modig et al.[80]

In an effort to determine if lower daily doses of toremifene were as effective as higher doses, toremifene treatment was evaluated by initially administering an oral loading dose: 120 mg on day 1, 60 mg on days 2 and 3, and thereafter 20 mg daily.[81] Of the 14 patients treated according to this schema, three achieved a PR (21%), and seven achieved stabilization of disease. No CRs were observed. In a multicenter study involving 38 patients with advanced disease, oral toremifene was administered at a dose of 240 mg/d.[82] An objective response rate of 68% was observed (CR 26%; PR 42%). This study suggested that higher doses of toremifene are more effective in inducing objective responses compared to low-dose toremifene (20 mg/d).

Toremifene has been directly compared with tamoxifen as first-line hormonal therapy in patients with advanced disease.[50,84-86] Three small randomized trials, lacking the statistical power to make a valid comparision between tamoxifen and toremifene, have been published. In a randomized trial comparing toremifene (40 mg/d orally) to tamoxifen (20 mg/d orally), Nomura et al[84] observed similar response rates and median time to disease progression in both treatment arms. Konstantinova and Gershanovich[85] reported on 47 patients randomized to one of two doses to toremifene (60 mg/d orally or 240 mg/d orally) or tamoxifen (40 mg/d orally). Patients treated with 60 mg/d of toremifene had a response rate of 50% compared with 35% in the higher-dose toremifene arm and 36% in the tamoxifen arm. In contrast, Stenbygaard et al[86] reported inferior response rates for patients treated with toremifene (240 mg/d orally) compared with tamoxifen (40 mg/d orally).

In a recently reported international trial,[50] 648 previously untreated, hormone receptor–positive or –unknown, metastatic breast cancer patients were randomized between tamoxifen (20 mg/d orally) or two different doses of toremifene (60 or 200 mg/d orally). Tamoxifen produced a response rate of 19% and a median survival of 32 months. Toremifene produced a response rate of 21% with the 60-mg dose and 23% with the 200-mg dose. Median survival of toremifene-treated patients was 38 months (60 mg/d) and 30 months (200 mg/d). The median time to disease progression was not statistically different between treatment arms.[50] Response rates, times to disease progression, and overall survival for patients on each arm were superior for ER-positive patients compared with ER-negative patients; however, no statistical difference in any of these end points was detected between treatment arms. Furthermore, quality-of-life assessments were not different between treatment arms. Toxicity was mild in all patients, but toremifene-treated patients experienced less nausea (26% *v* 37%). The data from this large trial[50] supports the use of toremifene as an alternative first-line therapy to tamoxifen in hormone receptor–positive, postmenopausal patients with advanced disease.

Toremifene appears to have cross-resistance to tamoxifen because overall response rates to toremifene after tamoxifen therapy in several phase II studies are low.[87-92] The largest experience was a multicenter trial reported by Vogel et al,[91] in which 102 perimenopausal or postmenopausal women with metastatic breast cancer refractory to tamoxifen received toremifene 200 mg/d. Patients in this trial were heavily pretreated, with 65% having failed chemotherapy and 72% having failed two or more hormonal therapies. Forty-nine percent of patients had visceral-dominant disease. The objective response rate was 5%, with only two patients achieving a CR. An additional 23% of patients maintained stable disease status for a median of 8 months.

Toremifene was observed to have an antitumor effect in mice with ER-negative uterine sarcomas and, as a result, a cytolytic effect independent of the ER was postulated.[46] In a small trial of nine patients with ER-unknown breast cancer the progressed after tamoxifen therapy, 33% of patients (three of nine) responded to toremifene (200 mg/d orally).[87] In an effort to confirm a mechanism of action independent of the ER, the Cancer and Leukemia Group B (CALGB) conducted a phase II trial to test the efficacy of high-dose toremifene (400 mg/d) in a population of patients with hormone receptor–negative metastatic disease with limited prior chemotherapy exposure.[93] Twenty patients were enrolled, but no objective responses were observed. These findings reaffirm that toremifene is primarily active in patients with hormone receptor–positive metastatic breast cancer.

Droloxifene

Droloxifene, or 3-hydroxytamoxifen (Fig 3), began development in Germany by Klinge Pharmaceuticals (Munich) in the late 1970s and subsequently by Fujisawa in Osaka, Japan. The drug has been tested for the treatment of metastatic breast cancer but is being developed by Pfizer (Parsippany, NJ) for the treatment of osteoporosis in postmenopausal women.

Laboratory studies. Droloxifene and its major metabolite N-desmethyldroloxifene[94] have a 10-fold higher binding affinity for MCF-7 (ER-positive) breast cancer cells than tamoxifen. Droloxifene inhibits estrogen-stimulated cell replication by arresting cells in G_0/G_1.[95] In addition, droloxifene appears to be a more potent inducer of TGF-β than either tamoxifen or toremifene.[95] Similarly, droloxifene inhibits insulin-like growth factor (IGF)-1–stimulated growth of MCF-7 cells.[96] In contrast, droloxifene does not inhibit the growth of the ER-negative cell line, MDA-MB-231. These findings confirm droloxifene is active through the ER.

Droloxifene shows antiestrogenic activity in the immature rat uterine weight test, but also causes a partial increase in uterine wet weight when administered alone.[96] The partial agonist action of droloxifene is only slightly less potent than tamoxifen[97]; however, droloxifene does maintain bone density in the ovariectomized rat.[98] Droloxifene shows antitumor activity in several rat and mouse models. Droloxifene inhibits the growth of the transplantable rat mammary tumor R3230AC, and both DMBA-induced[95,99] and N-nitrosomethyl urea (NMU)-induced[100] rat mammary tumors, but only ER-positive breast tumors transplanted into athymic mice.[101]

Toxicology. Droloxifene does not produce DNA adducts or hepatocellular carcinomas in male or female rats fed daily doses of 36 mg/kg.[95] One male and one female rat (2% total) developed a hepatocellular carcinoma after treatment for 24 months with 90 mg/kg/d. In comparison, tamoxifen produced hepatocellular carcinomas in 100% of animals after 36 mg/kg/d for 24 months.[95] Droloxifene is inactive in the ability to transform Syrian hamster embryo cells in vitro, whereas tamoxifen and 4-hydroxytamoxifen produce a significant level of transformation.[102]

Clinical pharmacology and endocrinology. Droloxifene is rapidly absorbed and excreted and does not appear to accumulate like tamoxifen and toremifene. Droloxifene can be monitored in serum using HPLC.[103,104] Under chronic dosing conditions, steady-state levels of parent drug were 83.5 ± 32.5 ng/mL (40 mg daily) and 146 ± 115.4 ng/mL (100 mg daily) with a half-life of 28 and 27 hours, respectively. Steady-state levels were achieved rapidly within 5 hours.[103]

There are several metabolites of droloxifene and all are present in serum as both free and glucuronide conjugates. This profile contrasts with tamoxifen, which does not have serum glucuronide metabolites.[103] Droloxifene causes a dose-related decrease in LH and FSH in postmenopausal patients.[105] There is only a modest decrease at the 20- and 40-mg dose daily, whereas there is a definite decrease at the 100-mg daily dose. Similarly, there is only a marginal increase in SHBG at the 20- and 40-mg daily dose, but a moderate increase occurs with 100 mg droloxifene daily. These conclusions have recently been confirmed in patients treated with 40 mg droloxifene daily.[104]

There have been numerous clinical trials evaluating droloxifene in patients with metastatic breast cancer.[106-112] The majority of patients on these trials had been previously treated with chemotherapy and/or hormone therapy. The daily dose of droloxifene ranged from 20 to 300 mg. Response rates ranged from 0% to 70%, with most responses occurring in perimenopausal or postmenopausal patients. Trials evaluating different doses of droloxifene have not convincingly demonstrated a dose-response effect.[109,111,112]

The largest clinical trial involving patients with metastatic breast cancer treated with droloxifene was recently updated by Rausching and Pritchard.[111] This phase II study compared droloxifene in doses of 20, 40, and 100 mg daily in postmenopausal women with metastatic, inoperable recurrent, or primary locoregional breast cancer who had not received prior hormonal therapy. Of 369 patients randomized, 292 were eligible and 268 assessable for response. Response rates were 30% in the 20-mg group, 47% in the 40-mg group, and 44% in the 100-mg daily group. Most responses occurred within 2 months of starting therapy. In all trials reported, droloxifene has been extremely well tolerated, with the most common toxicities cited being hot flashes, fatigue, and nausea.

Idoxifene

The drug was originally designed at the Cancer Research Campaign Laboratory in Sutton, Surrey, United Kingdom, and was offered to the pharmacutical industry through British Biotechnology (London, United Kingdom). Idoxifene is currently undergoing evaluation and development by SmithKline Beecham in the United Kingdom (London) and the United States (Philadelphia, PA).

Laboratory studies. Idoxifene has a binding affinity for the ER that is approximately twice that of tamoxifen, and this translates to a modest increase, compared with tamoxifen, in the ability to inhibit the growth of ER-positive, MCF-7 breast cancer cells in culture.[113] As would be expected with a compound that cannot be metabolically activated to the 4-hydroxy derivative,[56] idoxifene is a less potent (approximately 10-fold) antiestrogen in immature rat uterine weight tests.[113] However, idoxifene has less uterotropic activity when administered alone.[113]

Idoxifene demonstrates antitumor properties in the NMU-induced rat mammary carcinoma model in the 1- to 2-mg/kg dose range.[113] Although this range is somewhat higher than reported for tamoxifen, this would be expected from a drug that cannot be metabolically activated.

The investigators did show superior activity to tamoxifen at the 1-mg/kg dose.[113]

Toxicology. The drug was designed to avoid the toxicology problems with liver carcinogenicity produced by tamoxifen.[55,56] However, idoxifene has not been evaluated as a rat liver carcinogen. It is known that N-demethylation is the major metabolic route for tamoxifen; therefore, a pyrrolidino group was used to resolve liver toxicity through a reduction in formaldehyde production. Pyrrolidino tamoxifen (at half the dose) gives levels of DNA adducts as high as tamoxifen in rat liver.[114] Mice show interstitial hyperplasia in the ovaries and the expected decrease in uterine weights after 4 weeks of treatment with 25 to 50 mg/kg of daily idoxifene. A single-dose study in mice at 100 mg/kg shows no mortality or behavioral changes.[113] Tamoxifen produces similar actions in the mouse, but the metabolic prolifile in the mouse differs from the rat,[115] and mice do not produce liver tumors in response to tamoxifen.[1] Idoxifene is designed to be an agent that is metabolism-restricted, and as such requires careful evaluation for unusual or toxic routes of metabolism not previously noted with tamoxifen.

Clinical pharmacology and endocrinology. Preliminary studies in the laboratory with radioiodine 125 and 131 (^{125,131}I) idoxifene demonstrate no metabolism up to 48 hours after administration.[116] The studies are being used to establish a data base for the evaluation of ^{124}I idoxifene by positron emission tomography (PET) imaging during clinical studies. Idoxifene can be measured by HPLC,[117] but although 4′-hydroxyidoxifene is the most likely metabolite, it has not been reported in clinical studies. Idoxifene has an initial half-life of 15 hours and a terminal half-life of 23.3 days (ie, three times greater than tamoxifen). Idoxifene causes a modest decrease in LH and FSH but no increase in SHBG.[117]

Clinical evaluation. Only one clinical trial in humans has been reported with idoxifene. Coombes et al[117] reported the results of a phase I clinical trial in which 20 patients with advanced breast cancer (ER-positive or -unknown) were treated with one of four dose levels of idoxifene . The majority of patients previously received tamoxifen, second-line hormone therapy, and chemotherapy. PRs were observed in 14% of patients, and an additional 29% of patients had stable disease for 1.4 to 14 months. Toxicitiy was mild and not dose-related.

TAT-59

TAT-59 is a prodrug and is being developed by the Taiho Pharmaceuticals Company, Ltd, in Tokushima, Japan for the treatment of advanced breast cancer.

Laboratory studies. TAT-59 is active in inhibiting the growth of ER-positive, DMBA-induced rat mammary carcinomas.[118] The drug is converted to its dephosphorylated metabolite[118] that has high binding affinity for the ER (Fig 3).[119] The drug is active in inhibiting the growth of estrogen-stimulated, ER-positive breast cancer cells transplanted into athymic mice.[119,120]

Clinical evaluations. Clinical studies using TAT-59 for the treatment of advanced breast cancer have not been published.

PURE ANTIESTROGENS

Pure antiestrogens were first described in the mid-1980s.[41] The compound ICI 182,780 (ZM.182-780) is being developed by Zeneca Pharmaceuticals (Macclesfield, United Kingdom) for the treatment of advanced breast cancer after failure of long-term adjuvant tamoxifen therapy (Fig 4). Pure antiestrogens could also find application in gynecology and other nonmalignant conditions.

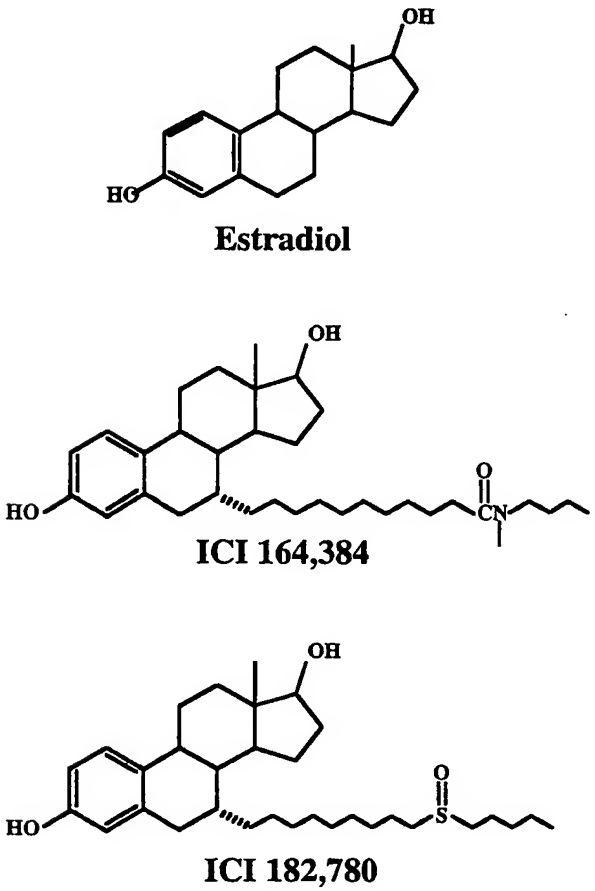


Fig 4. The formula of steroidal pure antiestrogens. The structure of estradiol is shown for comparison.

Laboratory Studies

ICI 182,780 is a competitive inhibitor of estrogen action that blocks estrogen binding to the ER[42] and by causes the destruction of the ER.[42,43] This pure antiestrogen is a potent inhibitor of the growth of MCF-7 cells (10^{-9} to 10^{-7} mol/L) and causes a more complete inhibition of growth compared with tamoxifen.[44] The ER-negative cell line, MDA-MB-231, is unaffected by pure antiestrogens.

ICI 182,780 is a potent and complete antiestrogen when given orally or subcutaneously to immature rats. Futhermore, ICI 182,780 can inhibit the partial estrogen-like effects of tamoxifen on the rat uterus.[44] This may be important because tamoxifen can eventually encourage the growth of MCF-7 breast cancer cells implanted into athymic mice.[121,122] Similarly, tamoxifen-stimulated endometrial carcinoma has been reported to grow in athymic animals.[123,124] Pure antiestrogens will inhibit the growth of tamoxifen-stimulated breast and endometrial tumors in the laboratory.[125,126] The antiestrogen ICI 182,780 will control the growth of tamoxifen-stimulated tumors for prolonged periods; however, growth eventually occurs. Preliminary studies[127] suggest that ICI 182,780–resistant tumors may be developed, but further work is required to describe the model in detail. Rats with established DMBA-induced tumors show a more rapid decrease in tumor size and uterine weight with a combination of the luteinizing hormone-releasing hormone (LHRH) analog, goserelin, and ICI 164,384 compared with goserelin alone[128] The combination may provide a more complete estrogen blockade that might eventually be valuable for the treatment of premenopausal patients with advanced disease.

Toxicology

There have been no reports of genotoxicity or carcinogenesis with ICI 182,780. Unlike estradiol or tamoxifen, the administration of ICI 164,384 to neonatal female rats did not accelerate the onset of puberty or lead to abnormalities in the development of the reproductive tract.[129]

Clinical Pharmacology and Endocrinology

There are no reports about the metabolism of ICI 182,780, but injection of 18 mg/d produces blood levels of 25 ng/mL after 1 week of treatment. ICI 182,780 is determined by radioimmunoassay.[130] Patients treated with ICI 182,780 for a few days have a significant decrease in the Ki67, progesterone receptor (PgR), and ER in their breast tumors.[130] ICI 182,780 has no effect on LH, FSH, or SHBG.[130]

Studies in both ovariectomized[131] and intact[132] adult female monkeys demonstrate a complete blockade of the uterotrophic effects of estradiol by ICI 182,780. The pure antiestrogens could find use in the treatment of endometrial disorders and endometrial carcinoma. A comparison with progestins in clinical trials might demonstrate fewer side effects with a pure antiestrogen.

Clinical Evaluations

Data from clinical trials evaluating the activity of pure antiestrogens are limited. DeFriend et al[130] assesssed the tolerance, pharmacokinetics, and short-term biologic activity of ICI 182,780 in 56 women with primary breast cancer. Patients were randomized to a control group (n = 19) in which no preoperative therapy was administered, or to one of two preoperative treatment groups (treatment group 1: 6 mg intramuscularly for 7 days, n = 21; treatment group 2: 18 mg intramuscularly for 7 days, n = 16). ICI 182,780 was not associated with any significant toxicity.

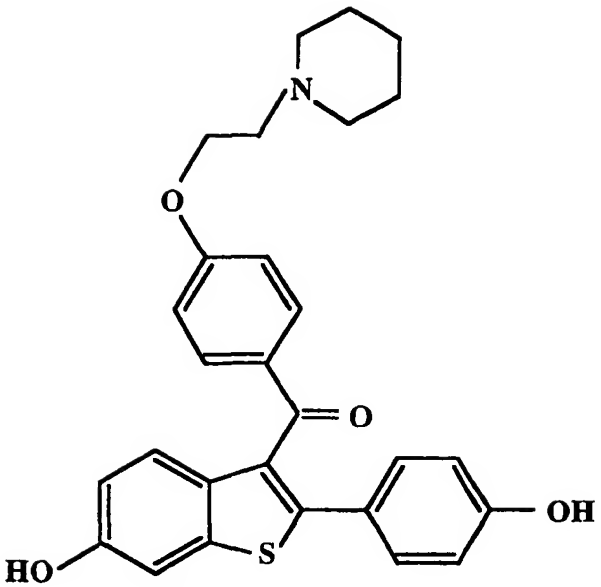
In a group of 19 tamoxifen-resistant, advanced breast cancer patients, Howell et al[45] reported a 37% PR rate using ICI 182,780 (250 mg/month intramuscularly). Soft-tissue, bone, and visceral sites of disease responded. In addition, 32% of patients maintained stable disease status. No significant toxicity was observed. The lack of cross-resistance with tamoxifen in 69% of patients suggests that ICI 182,780 may be useful as a first-line therapy in advanced disease or as a second-line therapy in advanced disease where tamoxifen has been previously used. The activity of pure antiestrogens in advanced disease suggests that they may also have efficacy in the adjuvant setting, although no data are available.

TARGETED ANTIESTROGENS

Raloxifene (originally called keoxifene) (Fig 5) is being developed by Eli Lilly Laboratories as a treatment for osteoporosis. The drug is referred to as a selective estrogen receptor modulator (SERM) that builds on the concept that targeted antiestrogens can be found to have estrogenic effects on the cardiovascular system and bone, but an antiestrogenic action on the breast and uterus.[3,133] Raloxifene is also being evaluated as an antitumor agent in ER-positive advanced breast cancer patients.

Laboratory Studies

Raloxifene has a binding affinity for the ER equivalent to that of estradiol.[27] The compound is a potent inhibitor of the growth of breast cancer cells in culture.[32] Raloxifene is an antiestrogen in the immature rat uterine weight test but has little agonist action on the uterus when administered alone. It is well established that raloxifene can maintain bone density in the ovariectomized rat,[36-39] but the drug also reduces circulating cholesterol.[38]



RALOXIFENE

Fig 5. Raloxifene, a specific estrogen receptor modulator in clinical trials to treat osteoporosis.

At low doses, raloxifene has weak antitumor activity in the DMBA-[33] and NMU-induced[35] rat mammary carcinoma models compared with tamoxifen. Raloxifene-stimulated breast tumors have not been described, but raloxifene partially inhibits the tamoxifen-stimulated growth of the human endometrial carcinoma, EnCa101, in athymic mice.[126]

Toxicology

No reports of DNA adducts or hepatocarcinogenesis have appeared with raloxifene.

Clinical Pharmacology and Endocrinology

An analytical method to determine raloxifene and its metabolites has not been published. The drug causes a decrease in low-density lipoprotein (LDL) cholesterol, but high-density lipoprotein (HDL) cholesterol remains unchanged during treatment with 200 and 600 mg daily.[134]

Clinical Evaluation

In the 1980s, a series of phase I studies of raloxifene was conducted in healthy male subjects. A once-daily oral dosing schedule was well tolerated, and acceptable blood levels were achieved. No clinically adverse events were detected, and there was evidence for an antiestrogenic effect.[135] Physicians at the M.D. Anderson Hospital reported the results of a phase II trial of raloxifene in female patients with metastatic breast cancer who were refactory to tamoxifen therapy.[136] Raloxifene 200 mg/d was administered orally in accordance with the highest dose given in phase I studies. Fourteen patients received raloxifene daily for up to 8 months. The drug was well tolerated with no significant clinical or laboratory abnormalities detected, but no objective responses were observed. As a result of the intense interest in raloxifene as a potential treatment for osteoporosis, an international clinical trial is underway to evaluate the activity of raloxifene in hormone receptor–positive, postmenopausal patients with metastatic breast cancer who have not received prior hormonal therapy or chemotherapy for metastatic disease. All laboratory studies strongly indicate that raloxifene would be an effective agent to control the growth of breast cancer.

The ability of raloxifene to maintain bone density in the rat[36-39] has encouraged the clinical testing of raloxifene as a treatment for osteoporosis. Large international clinical trials are currently underway to evaluate the effect of raloxifene on the progression of osteoporosis.

CONCLUSION

The successful development of tamoxifen has created numerous new opportunities for the development of drugs that could be applied throughout medicine. In this review, we have described the properties of several new agents being tested clinically, but it is important to stress that the current development of these new agents is focused on several different therapeutic goals. Although this fact makes a direct comparison of compounds difficult, it is useful to evaluate the available data in the areas that have

Table 1. A Comparison of Published Reports About the Toxicology and Application of New Antiestrogens

	Toremifene	Droloxifene	ICI 182,780; ICI 164,384	Raloxifene; Keoxifene
DNA adducts	No[51]	No[114]	—	—
Rat liver tumors	No[51,52]	No[95]	—	—
Endometrial cancer (laboratory)	—	—	Inhibit[3]	Inhibit[126]
Endometrial cancer (clinic)	—	—	—	—
Cross-resistance with tamoxifen	Yes[86,91]	—	No[45]	—
Advanced breast cancer	Yes[50]	Yes[111]	Yes[45]	—
Adjuvant therapy	—	—	—	—
Cholesterol (+) (laboratory or clinic)	Yes[137]	—	—	Yes[38,134]
Preserves bones (+) (laboratory)	—	Yes[98]	No[138]	Yes[36-39]

NOTE. No represents a report where a characteristic was not found, yes represents a report where a characteristic was found, and a dash indicates no published studies.

caused the most concern with tamoxifen. The principal areas of evaluation in the laboratory and clinic are listed in Table 1. Additional information is available within the individual pharmaceutical companies to justify current clinical trials with regulatory authorities; however, it is only possible to cite published data in the medical literature.

Overall, much progress has been made during the past decade; however, as illustrated in Table 1, there are still significant gaps in our knowledge about the efficacy and long-term safety of the new agents. A focused program of development in each of the targeted areas of therapeutics will result in the introduction of numerous new antiestrogens (SERMS) into clinical practice by the end of the 1990s.

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Review article

Preclinical data for Droloxifene

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Abstract

The new antiestrogen Droloxifene has a 10–60-fold higher binding affinity to the estrogen receptor (ER) compared to the related compound Tamoxifen. A similar relationship was found in growth inhibition studies which showed that Droloxifene inhibited the different ER positive human breast cancer cells more effectively than Tamoxifen, predominantly in drug concentrations which are found in humans during therapy. As another consequence of the high stability of the complex formed by Droloxifene binding to the ER, intermittent exposures with clinically relevant concentrations of Droloxifene brought about effective growth inhibition of human ER positive tumor cells even after short-term application. Droloxifene was found, like Tamoxifen, to block human breast cancer cells in G_1-phase of the cell cycle. Moreover, cell-cycle data confirmed the superior growth-inhibiting potency of Droloxifene compared to Tamoxifen. Droloxifene was also found to effectively induce expression of the negative growth factor TGF-β, to inhibit IGF-I stimulated cell growth and to prevent estrogen-stimulated proto-oncogene c-*myc* expression. Unlike Tamoxifen, Droloxifene is a potent inhibitor of protein biosynthesis in ER-positive breast cancer cells at physiologically relevant concentrations. Lower estrogenic and higher antiestrogenic effects on immature rat uterus indicate a higher therapeutic index for Droloxifene compared to Tamoxifen. In vivo, Droloxifene displayed increased growth inhibition of different tumors of animal (R3230AC and 13762) and human origin (T61). Furthermore, it was found that the two structurally similar drugs differ in their toxicologic characteristics in the following important respects: Droloxifene is devoid of any in vivo or in vitro carcinogenic or mutagenic effects, whereas Tamoxifen causes liver tumors in rats, induces DNA adduct formation in rats and hamsters and shows transforming activity in SHE-cells (Syrian hamster embryo fibroblasts). Considerably less toxicity and a lower level of intrinsic estrogenicity was observed even after maximum long-term exposure of different animal species to Droloxifene, in comparison with Tamoxifen. Therefore, it can be assumed that Droloxifene may represent an important step forward in the treatment of mammary carcinomas in women through its better tolerability and increased efficacy compared with Tamoxifen. For long-term adjuvant or preventive treatment of breast cancer, Droloxifene may well be the safer choice.

Keywords: Droloxifene; Preclinical data; Antiestrogen

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1. Introduction

The triphenylethylene antiestrogen Tamoxifen has become established as a first line endocrine treatment of hormone-dependent breast cancer [1]. However, Tamoxifen is not a pure antiestrogen but in fact shows a low antiestrogenic/estrogenic index [2] which may limit its therapeutic efficacy. In particular, Tamoxifens considerable estrogenic activities are thought to be the cause of observed tumor flares at the beginning of breast cancer therapy [3]. Furthermore, this estrogenicity may be involved in the development of secondary endometrial tumors which have been reported following long-term adjuvant treatment of breast cancer patients [4,5].

Because of its higher antiestrogenic potency, 4-hydroxytamoxifen has often been claimed to be the active metabolite of Tamoxifen. However, 4-hydroxytamoxifen is produced at only 1–3% of the concentration of its parent compound Tamoxifen in both patient serum and tumor tissues [6]. Here we review the pharmacology of Droloxifene, which, like 4-hydroxytamoxifen, may be classified as a high affinity antiestrogen [7] with fewer estrogenic and higher antiestrogenic activities, improved antitumor efficiency and toxicological advantages in comparison to Tamoxifen.

2. Binding affinity of Droloxifene to the estrogen receptor

The most important prerequisite for an efficient antiestrogen is high binding affinity to the estrogen receptor (ER) in order to displace 17β-estradiol from its binding site and to antagonize its action in different target organs (e.g. stimulation of growth of breast cancer cells).

Droloxifene and its major metabolite *N*-desmethyl-Droloxifene exhibited at least a 10-fold higher binding affinity to the estrogen receptor positive human breast cancer cell line MCF-7 when compared to Tamoxifen [8]. The superior binding affinity was confirmed in studies using estrogen receptor containing cytosols of juvenile rat or rabbit uterine tissues [9,10]. An investigation with estrogen receptor positive surgical specimens of human breast carcinomas indicated that the affinity of Droloxifene to the receptor was more

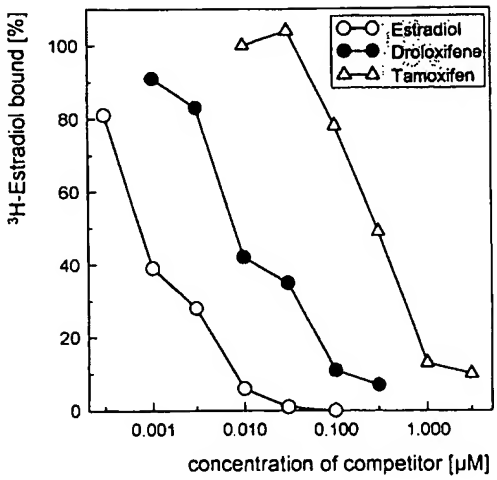


Fig. 1. Affinity of Droloxifene and Tamoxifen to the estrogen receptor of human MCF-7 breast cancer cells, determined in a competitive binding assay.

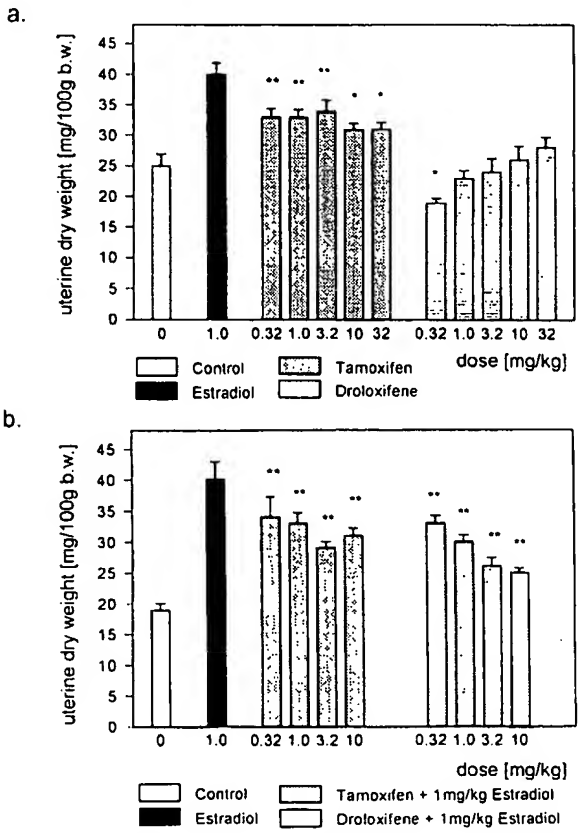


Fig. 2. Estrogenic (a) and antiestrogenic (b) activities of Droloxifene and Tamoxifen determined by the uterine weight test in immature rats (data from Kawamura et al. [11]). Statistical significance: $^*P < 0.05$, $^{**}P < 0.01$, compared to control group.

than 60-fold higher in comparison to Tamoxifen [8].

The IC_{50} value of Droloxifene for the displacement of 17β-estradiol from the ER was approximately 1×10^{-8} M (Fig. 1). As maximal blood concentrations of Droloxifene in humans reach approximately $1-4 \times 10^{-7}$ M, a sufficient antagonism of the ER mechanism should be guaranteed in breast cancer patients. These results demonstrated that the prerequisite for an effective antiestrogen, i.e. high binding affinity to the ER, is better fulfilled by Droloxifene than by Tamoxifen.

3. Estrogenic/antiestrogenic activity

Antiestrogens are compounds which prevent estrogens from expressing their effects on estrogen target tissues. Thus, they antagonize a variety of estrogen-dependent processes, including uterine growth of hormone-dependent breast tumors. However, most antiestrogens are not pure antagonists, but usually show some estrogenicity themselves. In the following experiments (Fig. 2) agonistic (intrinsic estrogenic activity) and antagonistic activities both were estimated by the influence of Droloxifene and Tamoxifen on uterine growth (change of uterine weight) in immature rats [9–11].

Immature rats received daily oral doses of Droloxifene and Tamoxifen, respectively, for three consecutive days. The estrogenic activity of the test compounds was estimated by the increase in uterine weight (uterotropic effect) while the antiestrogenic effect was tested by the reduction of uterine weight in the presence of 1 mg/kg estradiol (antiuterotropic effect). Droloxifene increased the uterine weights of immature rats by much less than the reference drug Tamoxifen. Thus, Droloxifene has a lower unwanted estrogenic effect in rats than Tamoxifen. This result is in line with another finding related to estrogenic activity, i.e. the lowering effect of Droloxifene on serum lipids of rats, which is weaker than that of Tamoxifen [unpublished results; [12]].

The antiestrogenic potency of Droloxifene,

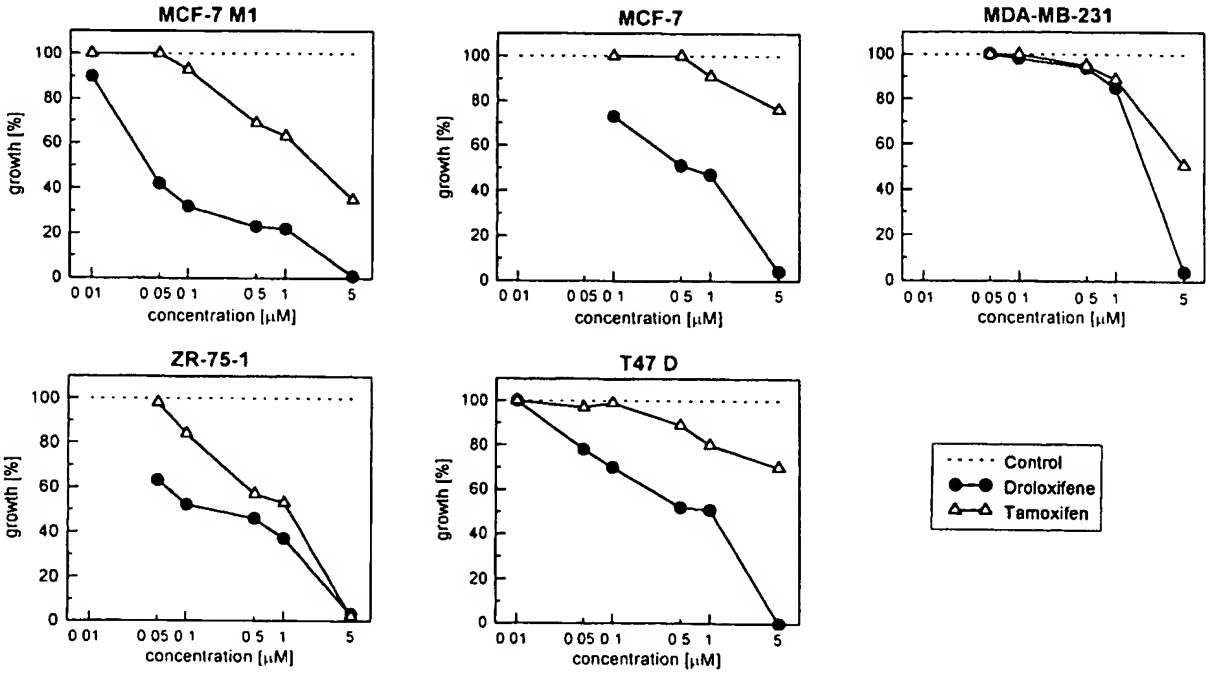


Fig. 3. Effect of Droloxifene and Tamoxifen on the growth of various human breast cancer cell lines in vitro.

tested by the reduction of uterine weight in estradiol-treated juvenile rats, was higher than that of Tamoxifen. This result can be clearly explained by the higher binding affinity of Droloxifene to the estrogen receptor.

In conclusion, Droloxifene had a lower estrogenic (immature rats) and a higher (mature rats) antiestrogenic effect on the uterine weight of rats than Tamoxifen. Therefore, the agonist versus antagonist index of Droloxifene is superior to that of Tamoxifen.

4. Effects on human tumor cell growth in vitro

4.1. Effect of Droloxifene and Tamoxifen on growth of various human breast cancer cells after continuous treatment

The in vitro antiproliferative effect of Droloxifene and Tamoxifen were tested using the following human breast cancer cell lines: firstly, MCF-7, MCF-7 M1, ZR-75 and T-47D cells, which all contain both estrogen (ER) as well as progesterone receptors (PgR); and secondly, MDA-MB-231 cells, which lack ER and PgR, and, therefore, enable the determination of an effect which is independent of the ER mechanism.

The influence of DROL and TAM on the growth of the different breast cancer cells was determined by incubation of the cells with different drug concentrations for 6 days. Then, the antiproliferative effect of both drugs was determined by: (i) electronic cell counting; (ii) measuring cellular DNA; or (iii) measuring [^{3}H]uridine incorporation into cellular RNA. All these methods provided similar results. The results shown below are based on the determination of total DNA content of culture dishes by the Burton method (Fig. 3).

Droloxifene inhibited the growth of the ER positive breast cancer cells, i.e. MCF-7, MCF-7 M1, ZR-75 and T-47D more effectively than Tamoxifen in the tested concentration range from 5 μM to 0.05 μM [13,14]. Moreover, it should be particularly noted that at 0.1 μM, the approximate serum level of both drugs in humans, Droloxifene showed a much higher antiproliferative activity in these cancer cells than did Tamoxifen (see charts in Fig. 3). The differential antiproliferative effects of both antiestrogens on the different cells correlated well with their cellular ER concentration, i.e. a high cellular ER content was paralleled by high antiproliferative activity.

From the tests with the ER negative cell line MDA-MB-231, we were able to differentiate which antiproliferative concentration is independent of the ER mechanism. Fig. 3 shows that the growth of the MDA-MB-231 cells could only be inhibited with drug concentrations exceeding 1 μM. Thus, with drug concentrations obtained in human serum during therapy ($\sim$0.1 μM), the antiproliferative effects of Droloxifene or Tamoxifen should be dependent mainly on the ER mechanism.

In MCF-7 M1 cells, Droloxifene was further compared to Tamoxifen and to another antiestrogen, Toremifene [15]. The IC_{50}-value for growth inhibition was about 0.05 μM with Droloxifene, but at least 20 times higher when Tamoxifen or Toremifene, which proved equally potent, were used (Fig. 4).

The superiority of Droloxifene in comparison to Tamoxifen and Toremifene with regard to tumor cell-growth inhibition is in good agreement with the higher binding affinity of the substance to the ER relative to Tamoxifen and Toremifene.

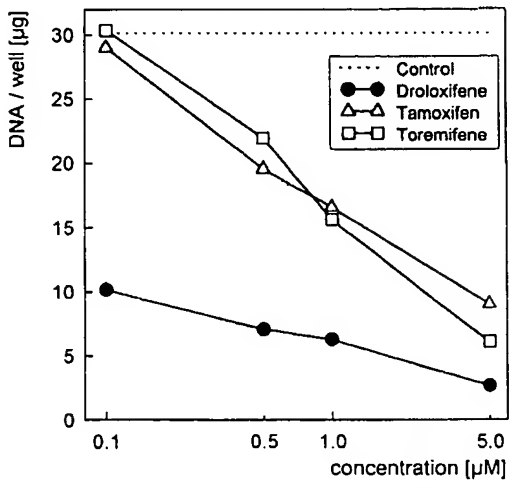


Fig. 4. Comparison of the effects of Droloxifene, Tamoxifen and Toremifene on the growth of MCF-7 M1 cells.

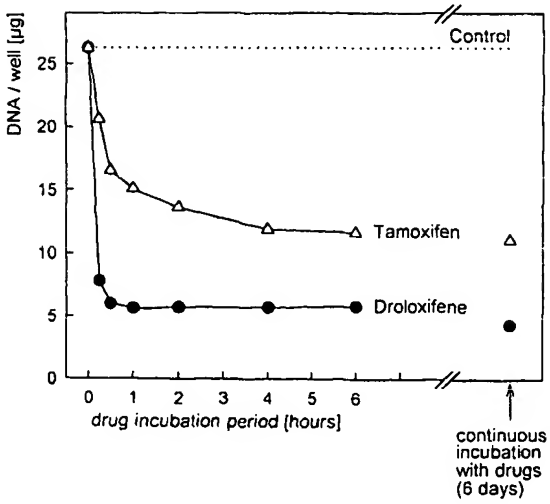


Fig. 5. MCF-7 M1 cell growth determined on day 6 after various treatment periods with either 0.5 µM Droloxifene or 0.5 µM Tamoxifen.

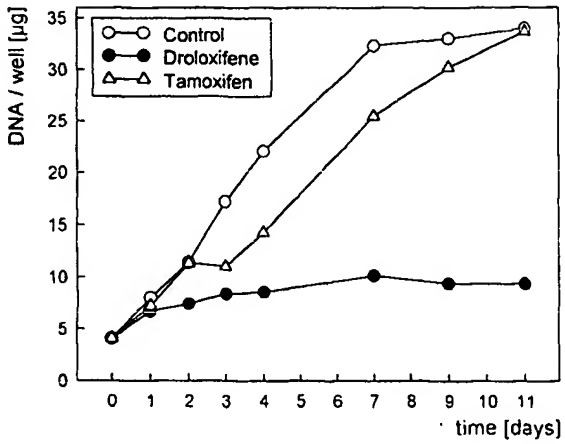


Fig. 6. Comparison of MCF-7 M1 cell-growth inhibition after a single 2-h treatment period with either 0.5 µM Droloxifene or 0.5 µM Tamoxifen.

4.2. *Inhibition of growth of human breast cancer cells by intermittent exposures to Droloxifene*

Droloxifene is predisposed by its pharmacokinetic characteristics, to be suitable for intermittent therapy regimens which may prevent or delay the development of resistance, and/or the induction of endometrial carcinoma, which have been observed during adjuvant treatment of breast cancer patients with Tamoxifen. Therefore, the growth inhibiting potency of continuous versus short-time incubations with Droloxifene was investigated in the ER positive human mammary carcinoma cell line MCF-7 M1 [16,17].

In a first experiment (Fig. 5), the antiproliferative effect of various short-time incubations of MCF-7 M1 cells with Droloxifene was compared with that of Tamoxifen. The maximum growth inhibition achieved by 0.5 µM Droloxifene was far more pronounced than that achieved by equimolar Tamoxifen. For maximum effect, a 1-h exposure to Droloxifene was sufficient, whereas a 3-h incubation period was needed for Tamoxifen.

The prolonged growth inhibition after a short incubation with Droloxifene was demonstrated when cell growth was recorded over 11 days in response to a single 2-h treatment with various concentrations of the antiestrogen (Fig. 6). Complete growth inhibition, and decreased DNA content in the culture dishes was observed after a single 2-h application of as little as 0.1 µM Droloxifene, a concentration which is easily obtained in human serum.

The suitability of Droloxifene for intermittent therapy was confirmed when its antiproliferative effects were investigated over 3 weeks. Two-hour treatment intervals with 0.5 µM Droloxifene every third day completely inhibited cell growth (Fig. 7).

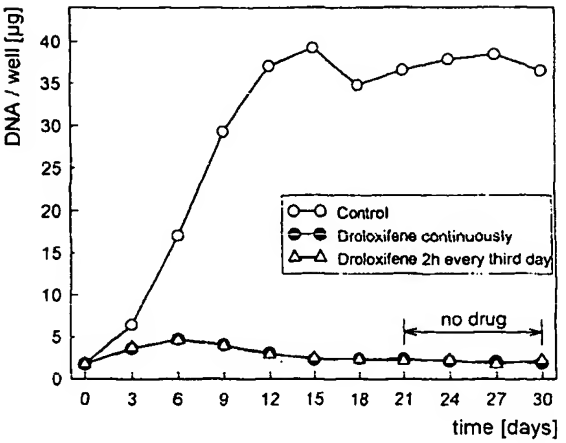


Fig. 7. Comparison of MCF-7 M1 cell-growth inhibition by continuous and intermittent treatment with 0.5 µM Droloxifene. Drug treatment was discontinued after 21 days.

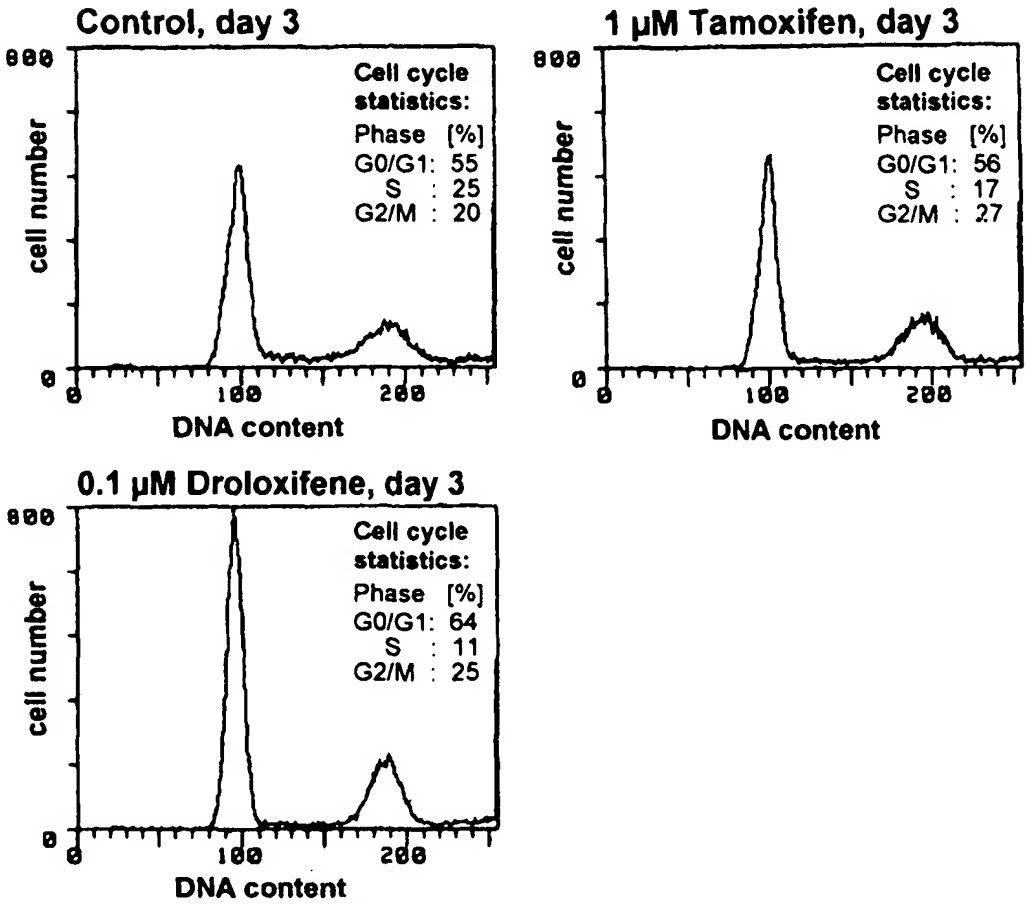


Fig. 8. Cell-cycle distribution of MCF-7 M1 cells incubated for 3 days either without drug (top), or with 0.1 µM Droloxifene (centre), or with 1 µM Tamoxifen (bottom).

When antiestrogen treatment was stopped after 21 days, and cell growth was recorded for 30 days after plating, no onset of proliferation occurred.

Because of its rapid uptake and sustained cell-growth inhibition after a short application, Droloxifene may be the first antiestrogen suitable for intermittent hormonal therapy regimens in breast cancer patients. This view is supported by the results of a preliminary clinical trial [18].

4.3. Cell cycle specificity of Droloxifene inhibition of human breast cancer cells

Tamoxifen is known to inhibit the growth of ER positive cells specifically in the G_1-phase of the

Table 1
Relative S-phase fractions in % of control of MCF-7 M1 cells incubated with various concentrations of droloxifene or tamoxifen

Time (days)	Droloxifene			Tamoxifen		
	1 µM	0.5 µM	0.1 µM	1 µM	0.5 µM	0.1 µM
1	72	65	70	80	90	88
2	39	42	50	69	73	104
3	28	32	44	68	76	104

cell cycle [19]. Because Droloxifene was supposed to act in a similar way, the cell cycle specific effects on the MCF-7 M1 cell line of Droloxifene and Tamoxifen, were compared. Cell cycle analysis was performed by flow cytometry after staining cellular DNA with propidium iodide [20,21].

The results shown below (Fig. 8) indicate that both Droloxifene and Tamoxifen inhibited the proliferation of MCF-7 M1 cells in the G_1-phase of the cell cycle. Over 3 days, the number of cells in S-phase were reduced in a concentration-dependent fashion (Table 1). Droloxifene was more effective than a ten times higher concentration of Tamoxifen. The G_0/G_1-arrest is in contrast to the action of cytotoxic chemotherapeutic agents like doxorubicin and vincristine, and indicates that growth inhibition by Droloxifene is completely different from that of cytotoxic drugs. These cell cycle data again confirm that Droloxifene is much more effective in inhibiting cell proliferation than Tamoxifen.

4.4. Induction of transforming growth factor β (TGF-β) by Droloxifene

TGF-β belongs to a polypeptide family of growth factors which is involved in cellular proliferation, differentiation and development. It is produced by normal and transformed cells. TGF-β has different effects in different tissues, and we will refer only to the interactions between TGF-β and antiestrogens in breast cancer cell lines. In this context, the most important facts were published by Knabbe et al. [22] as follows:

1. TGF-β inhibits growth of the ER-positive human breast cancer cell line MCF-7, as well as ER-negative MDA-MB-231 cells.
2. Growth inhibitory antiestrogens like Tamoxifen and glucocorticoids strongly stimulate the secretion of TGF-β in MCF-7 cells.
3. Growth stimulatory estrogens and insulin decrease the secretion of TGF-β in MCF-7 cells.
4. Antiestrogen induction of TGF-β can be completely reversed by the addition of excess estrogens.

Thus, the inhibitory effect of an antiestrogen on breast cancer cells may be influenced by the amount of TGF-β which is induced and by the extracellular estrogen concentration. As TGF-β may be important for the growth inhibition of breast cancer cells, it was of interest whether Droloxifene was able to stimulate TGF-β secretion in MCF-7 cells as efficiently as the reference drug Tamoxifen and the new antiestrogen Toremifene.

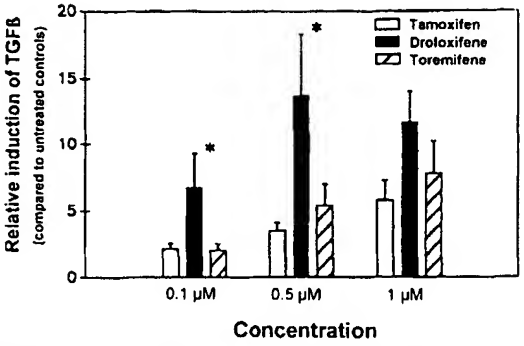


Fig. 9. Induction of TGF-β secretion by Droloxifene, Tamoxifen and Toremifene, measured by the determination of receptor reactive TGF-β in 48-h conditioned medium (data from Knabbe et al. [23]).

Droloxifene proved to be a very potent inducer of TGF-β secretion, whereby the maximum effect was already reached at 5×10^{-7} M [23]. Furthermore, the maximal effect of Droloxifene was by far higher than that of Tamoxifen and Toremifene (Fig. 9).

Furthermore, it was demonstrated that a single exposure of Droloxifene over 4 h was sufficient to induce a TGF-β secretion in MCF-7 cells 3–5 days later which was indistinguishable from the secretion rate obtained under continuous exposure (5 days). In contrast, a single exposure to Tamoxifen did not seem to have this long lasting effect on TGF-β secretion (Fig. 10).

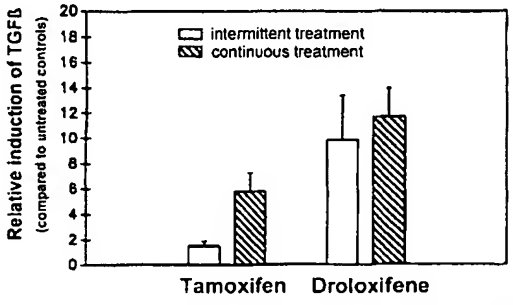


Fig. 10. Induction of cellular TGF-β secretion by intermittent versus continuous treatment of MCF-7 cells with 0.5 µM Droloxifene or 1 µM Tamoxifen (data from Knabbe et al. [23]).

In the patient's sera, Droloxifene concentrations were found to be around $1-4 \times 10^{-7}$ M during therapy. Such concentrations may be assumed to induce enough TGF-β in breast tumors to play an important role for the overall antitumor effect of Droloxifene and can be assumed to contribute to the superiority of Droloxifene over Tamoxifen and Toremifene.

4.5. Interaction of Droloxifene with the insulin-like growth factor Type I (IGF-I)

Insulin-like growth factor I (IGF-I), also referred to as somatomedin C, is a more potent mitogen for breast cancer cells in vitro than estradiol [24]. Therefore, in patient tissue, IGF-I derived from endocrine or paracrine origin may, in addition to estrogens, stimulate the growth of breast cancer cells [25].

It is well known that antiestrogens can block the growth stimulating effect of estrogens. However, the effect of antiestrogens on IGF-I induced cell proliferation was not known. Therefore, it was investigated whether Droloxifene and its reference drug Tamoxifen can influence the mitogenic effect of IGF-I on the human breast cancer cell line MCF-7 [26].

Both antiestrogens, Droloxifene and Tamoxifen, inhibited IGF-I stimulated growth of MCF-7 cells in a concentration-dependent fashion (Fig. 11). However, Droloxifene was more effective than Tamoxifen. As Droloxifene also blocks estrogen-stimulated proliferation of breast cancer cells more effectively, the overall efficacy of Droloxifene in the treatment of human breast tumor could be significantly higher than that of Tamoxifen.

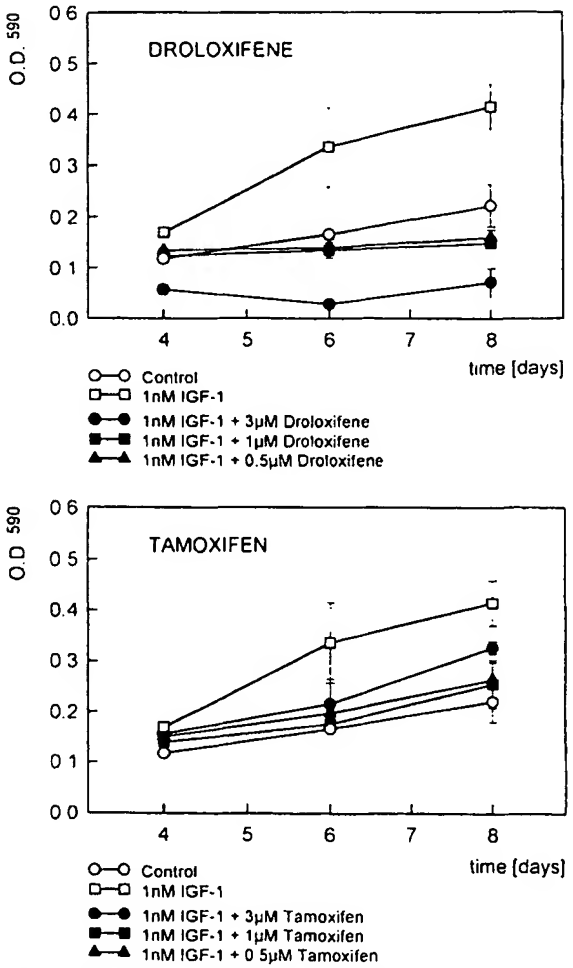


Fig. 11. Stimulation of MCF-7 cell-growth by IGF-I and its inhibition by antiestrogen treatment (data from Wosikowski et al. [26]).

4.6. Influence of Droloxifene on the proto-oncogene c-myc

Expression of the proto-oncogene c-*myc* has been described to play a role in the regulation of cell-cycle duration [27]. Because estrogen stimulation of breast cancer cells is known to increase c-*myc* levels, the influence of Droloxifene and of Tamoxifen was investigated.

Experiments by Wosikowski et al. [26] confirmed a significant increase in the expression of c-*myc* mRNA in MCF-7 cells detected by Northern Blot analysis as early as 15 min after administration of the estrogenic stimulus, with peak levels obtained after 60 min. However, when the cells were pretreated for only 30 min with 1×10^{-6} M Droloxifene before stimulation with estradiol, severe depression of estrogen inducible c-*myc* mRNA was detected; longer preincubation times had no additional effects. In contrast, preincubations with 1×10^{-6} M Tamoxifen did not substantially lower estradiol induced c-*myc* expression.

These results confirmed the rapid action and high antiestrogenic potency of Droloxifene. Effective prevention of estrogen-stimulated c-*myc* expression indicates that Droloxifene acts on an

early event in the regulation of cell proliferation. Also in this regard, Droloxifene is superior to Tamoxifen.

4.7. Influence of Droloxifene on protein synthesis of human breast cancer cells

To prove whether Droloxifene has an effect on cellular protein synthesis and to differentiate whether this effect was dependent on the estrogen receptor mechanism, an estrogen receptor positive (MCF-7) and an estrogen receptor negative (MDA-MB-231) human breast cancer cell line were used [28,29].

Droloxifene concentrations between 3×10^{-8} M and 1×10^{-6} M inhibited protein synthesis in MCF-7 cells under serum-free conditions dose-dependently with similar efficacy to cycloheximide, a well known inhibitor of eukaryotic protein synthesis; equimolar concentrations of the reference drug Tamoxifen as well as of Toremifene, showed no detectable effect (Fig. 12). In MDA-MB-231 cells only cycloheximide was able to reduce protein synthesis at submicromolar concentrations (data not shown). This result confirms the estrogen receptor dependency of protein synthesis inhibition by Droloxifene, and again demonstrates superiority of Droloxifene over Tamoxifen and Toremifene.

5. Effects on tumor growth in vivo

As demonstrated above, Droloxifene reduced the growth of different human breast cancer cell lines very effectively and more efficiently than Tamoxifen. Therefore, it was investigated whether this antitumor activity of Droloxifene could also be reproduced in vivo. Because animal models have a limited predictive value for drug action in humans due to their different metabolism, different animal systems were used in order to improve the prediction.

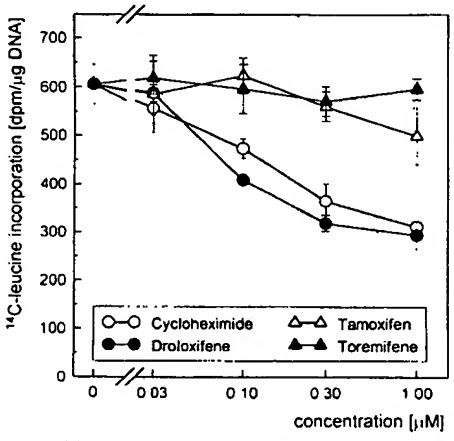


Fig. 12. Inhibition of protein synthesis in MCF-7 cells determined by radioactive leucine incorporation after treatment with antiestrogens or cycloheximide.

5.1. Tumors R3230AC and 13762 in Fischer 344 rats

The mammary adenocarcinomas R3230AC and 13762 grow rapidly in Fischer 344 rats. The growth of both tumors is estrogen-dependent and can be impaired by antiestrogen treatment. We compared the antitumor effects of identical doses of Droloxifene and Tamoxifen after daily oral application (6 days/week) in these two systems.

Fig. 13 (top left) indicates the growth inhibition of two different doses of Tamoxifen and Droloxifene, respectively, on tumor R3230AC in Fischer rats [10]. The median of tumor area per rat was reduced more effectively by Droloxifene than by Tamoxifen at either dose. The lower Tamoxifen concentration (1.5 mg/kg) seemed to be without effect. Tamoxifen (9.0 mg/kg) and 1.5 mg/kg Droloxifene both reduced tumor growth to the same extent.

As shown in Fig. 13 (top right), 9 mg/kg Tamoxifen could only delay the growth of the tumor 13762 slightly. In contrast, the same dose of Droloxifene resulted in a distinct reduction in the proliferation of this mammary tumor.

The fact that the growth inhibitory effect of both drugs was more pronounced in the R3230AC rat tumor model may be due to the high mutation frequency of the 13762 adenocarcinoma. Nevertheless, the results with the 13762 tumor clearly confirm the superiority of Droloxifene compared to Tamoxifen, which was found in the R3230AC model.

5.2. DMBA-induced tumors in Sprague–Dawley (SD) rats

It is generally known that antiestrogens elicit a high regression rate of DMBA-induced mammary tumors. In contrast to the R3230AC mammary tumor, the DMBA tumor shows a strictly ovarian-

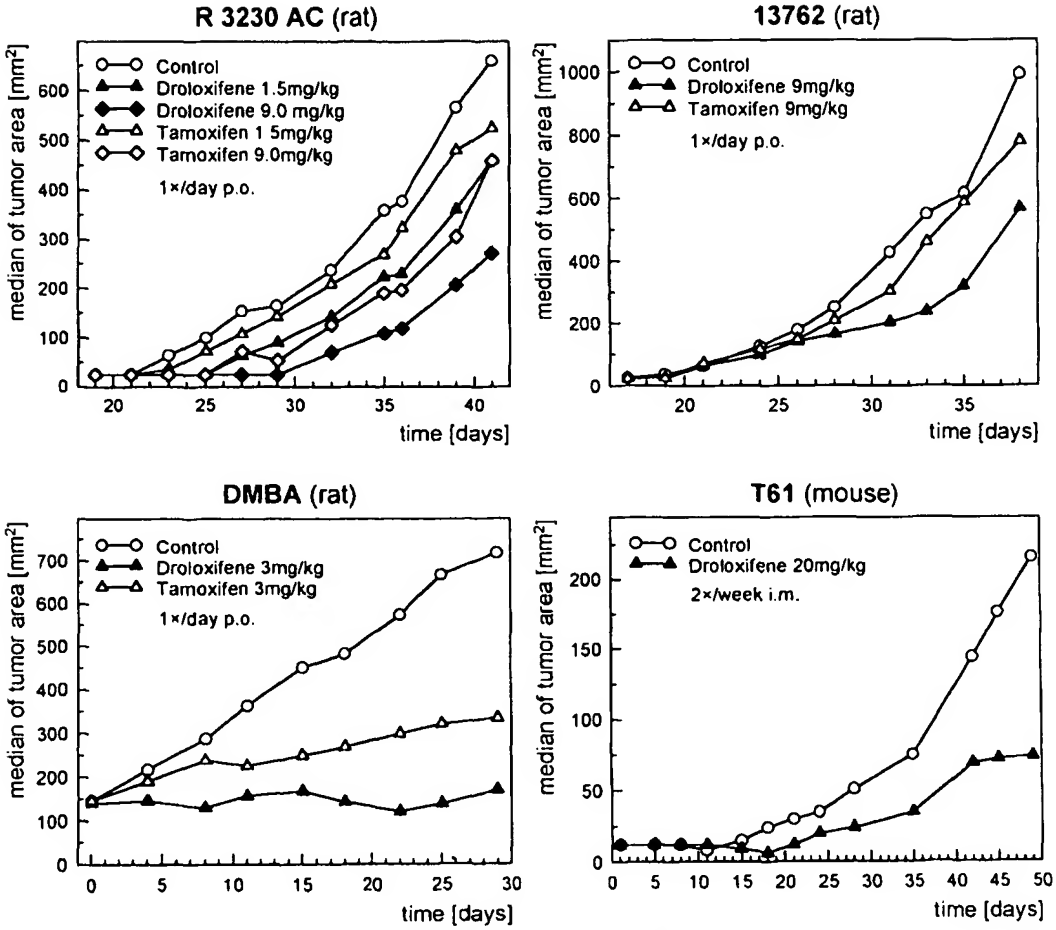


Fig. 13. Effect of Droloxifene and Tamoxifen on different tumors in rats and mice.

dependent growth. Thus, DMBA tumors regress after ovariectomy or antiestrogen administration. In our study the antitumor effects of daily oral administration to tumor bearing SD rats (6 days a week) of 3 mg/kg Droloxifene and Tamoxifen, respectively, were compared [30]. Fig. 13 (bottom left) shows that Droloxifene and Tamoxifen both decreased the growth of this tumor very effectively. However, it can be observed that the antitumor effect was more pronounced with Droloxifene in comparison to Tamoxifen.

A higher efficacy of Droloxifene in the treatment of rat mammary tumors was also demonstrated in other laboratories [31].

5.3. Tumor T61 in nude mice

The activity of Droloxifene was investigated in

Table 2
Relative affinity of the metabolites of Droloxifene to the estrogen receptor, compared to their growth inhibitory potency on MCF-7 M1 cells at a concentration of 0.1 μM, relative to Droloxifene

Compound	Receptor affinity (%) relative to DROL	Growth inhibition (%) relative to DROL at 10^{-7} M
Droloxifene	100	100
K21106E	87	100
K21157E	117	93
K21158E	23	84
K21159E	73	0
K21160E	50	20
K21161E	0.5	0
Tamoxifen	5	16

nude mice with transplants of the human breast cancer T61, which expresses a high estrogen receptor (ER) content. Droloxifene was given i.m. to the mice in a dose of 20 mg/kg, twice a week [32]. Tamoxifen was not included in this study, because, in comparison to Droloxifene, it shows no antiproliferative activity on the T61 tumor, as observed by Fukutomi et al. [33].

As demonstrated in Fig. 13 (bottom right) the administration of Droloxifene resulted in a significant inhibition in growth of the human breast cancer T61. This result was confirmed by others [33].

In conclusion, the results show that Droloxifene is able to reduce the growth of a variety of breast tumors of animal (R3230AC, 13762 and DMBA) and human (T61) origin. The superiority of Droloxifene over Tamoxifen, as observed in vitro in breast cancer cell lines, was also demonstrated in vivo.

6. Metabolism route of Droloxifene in different species including humans and pharmacological activity of the metabolites

For a better understanding of the in vivo activity of Droloxifene in humans and different animal species, it was of interest to know the metabolization pattern in the different species and to test the pharmacological activity of the different metabolites in comparison to the parent compound Droloxifene. The biological activity of all the metabolites found was determined by measuring their binding affinity to the estrogen receptor and by testing their potency in inhibiting growth of the well known estrogen receptor positive human breast cancer cell line MCF-7.

The following metabolites were detected in humans [34]: droloxifene-glucuronide (K21161E), *N*-desmethyl-droloxifene (K21106E), 4-methoxy-droloxifene (K21160E) and the side-chain-hydroxy-derivative of Droloxifene (K21158E). With the exception of the glucuronide K21161E ($IC_{50} = 5.8 \times 10^{-6}$ M), all other metabolites bound to the ER with approximately equal or somewhat less affinity (IC_{50} from 2.7×10^{-8} M to 1.2×10^{-7} M) compared to their parent compound Droloxifene ($IC_{50} = 2.7 \times 10^{-8}$ M). At clinically relevant serum concentrations of Droloxifene (approximately $1-4 \times 10^{-7}$ M) all but K21161E reduced the growth of MCF-7 cells to the same extent or somewhat less compared to Droloxifene.

Generally, metabolization was very low in the human samples, all metabolites except droloxifene-glucuronide appearing at less than 5% of the parent compound. Droloxifene seems to be efficiently inactivated and excreted by glucuronidation. Two additional metabolites, 3-methoxy-4-OH-tamoxifen (K21159E) and *N*-oxide-droloxifene (K21157E), were found in rats and mice, but could not be detected in the human samples. It is important to note that Droloxifene was metabolized into K21159E at a very high rate (>40%) in the mouse and to a lower rate (<20%) in the rat. This metabolite is of particular interest as it has a high binding affinity to the ER ($IC_{50} = 3.7 \times 10^{-8}$ M), but practically no antitumor activity on human breast cancer cells. As this inactive metabolite competes for the estrogen receptor with all other active metabolites, the antitumor activity of Droloxifene should be underestimated in mice compared to rats, and even more so when compared to humans.

7. Droloxifene induces no hepatocellular proliferative lesions in rat liver in contrast to Tamoxifen

Long-term toxicological studies were carried out in rats. As is the case in humans, antiestrogens have an antagonistic effect on this species, at least in low doses. Intrinsic estrogenicity is prominent after the administration of high doses, highlighting the agonistic effects of the antiestrogens as the predominant feature [34].

In a 6-month toxicity study 2, 20 and 200 mg/kg/day Droloxifene was compared with 0.6, 6, and 60 mg/kg/day Tamoxifen. After 6 months of treatment, there was no evidence of histopathological changes in the liver of any animals exposed to Droloxifene, while the different stages of the development of hepatic carcinoma from preneoplastic (clear, acidophilic and basophilic) changes up to anaplastic hepatomas were encountered in all rats treated with 60 mg/kg/day Tamoxifen. Moreover, trabecular and glandular tumor types, which could be classified as adenocarcinomas, developed in acidophilic and basophilic nodules.

Table 3
Incidence of hepatic proliferative lesions in rats treated with Droloxifene or Tamoxifen for a period of 24 months

	Males							Females						
	Placebo	Diet	Droloxifene				Tamoxifen	Placebo	Diet	Droloxifene				Tamoxifen
Dosage (mg/kg/day)	0	0	4	12	36	90	36	0	0	4	12	36	90	36
No. of animals examined	50	49	49	49	49	49	50	50	50	50	50	50	50	50
Basophilic foci	15	27	0	0	0	1	2	43	45	0	0	0	2	26
Hepatocellular adenoma	8	7	2	0	0	0	50	2	1	0	1	0	1	25
Hepatocellular carcinoma	0	0	1	0	0	1	49	0	0	1	1	0	1	50
Cholangioma	0	0	0	0	0	0	8	0	0	0	0	0	0	17

Table 4
Summary of 24-h serum concentrations (ng/ml) of Droloxifene, Tamoxifen and their respective metabolites at the end of the treatment period

Treatment (mg/kg)	Parent compound	*N*-Desmethyl-metabolite	4-Hydroxy-metabolite	4-Methoxy-metabolite	3-Methoxy-4-hydroxy-metabolite	*N*-Oxide-metabolite	Di-*N*-desmethyl-metabolite	Total drug plus metabolites
Droloxifene								
4	27.8	3.8	—	1.9	28.9	0.1	—	62.5
12	93.6	13.7	—	4.3	64.3	2.9	—	178.8
36	408.1	72.7	—	12.8	231.1	15.8	—	740.5
90	1056.0	235.1	—	20.0	377.5	43.3	—	1731.9
Tamoxifen								
36	352.6	230.3	4.5	—	—	77.2	194.8	859.4

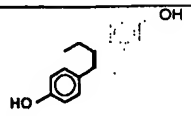
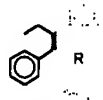
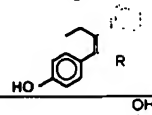
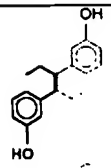
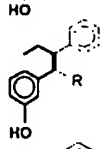
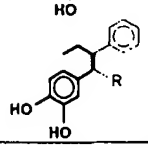
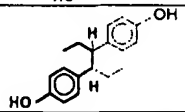
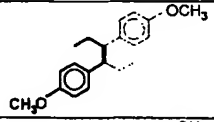
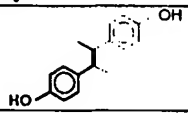
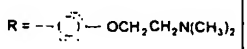
Compound	Significant Morphological Transformation
DES 	yes
Tamoxifen 	yes
4-Hydroxy-Tamoxifen 	yes
3,3'-DES 	no
3-Hydroxy-Tamoxifen (Droloxifene) 	no
3,4-Dihydroxy-Tamoxifen 	no
Hexestrol 	no
DES-dimethylester 	no
DMS 	no
	

Fig. 14. In vitro transformation of Syrian hamster embryo cells by stilbene estrogens and triphenylethylene-type antiestrogens.

Significant changes in nuclear sizes, nucleus/plasma relation and an increase in mitotic rates characterized these proliferations. These changes were more pronounced in females than in males. Convincing evidence of the autonomous reversible feature of the nodular changes in the hepatic tissue and unequivocal development from bile duct proliferation to adenoma of the bile duct cells were apparent at the end of the 6-week reversibility period.

The 6-month study was followed by a 24-month carcinogenicity study. Droloxifene doses ranged from 4 to 90 mg/kg/day and a 36 mg/kg/day Tamoxifen group was included. The carcinogenicity of

Tamoxifen observed in the 6-month study was confirmed in the carcinogenicity study when all animals, treated with 36 mg/kg/day Tamoxifen, developed hepatoproliferative lesions with 100% incidence of hepatocellular carcinomas (Table 3).

In contrast to this, no evidence of tumorigenic effects of Droloxifene on the liver has been noted. Moreover, Droloxifene exerted an inhibitory effect on basophilic foci, which appeared spontaneously in the control groups.

Our results are in agreement with those of Hirsimäki et al. [36], Hard et al. [37], Greaves et al. [38] and Williams et al. [39], who observed a tumorigenic effect of Tamoxifen in rats treated with 48 mg/kg, 11.3 mg/kg, and 5 mg/kg.

Additionally, Tamoxifen induced a time and dose-dependent accumulation of DNA-adducts in rodent livers [40,41], which could lead to genetic mutations, and a significant dose-dependent increase in micronucleus formation in the human lymphoblastoid MCL-5 cell line [41]. DNA from Droloxifene-treated rats, however, did not contain any adduct spots other than those detected in the DNA of control rats [41].

According to the transformation test with Syrian hamster embryonic fibroblasts (SHE), performed by Metzler et al. [42], the carcinogenic action may be due to the DES-like structure of Tamoxifen and 4-OH Tamoxifen (metabolite of Tamoxifen). Metzler et al. and McLachlan et al. [43] were able to prove that the genotoxicity of these two compounds, as well as that of DES is dependent upon two structured features: a 4-OH group at the aromatic ring and the stilbene double bond (Fig. 14). A 3-OH group, however, prevents the transformation of SHE-cells, therefore, neither 3,3 DES nor 3-OH Tamoxifen (Droloxifene) cause cell transformation.

Thus, the comparative long-term toxicity studies of Droloxifene and Tamoxifen have demonstrated that Tamoxifen has an unequivocal carcinogenic effect, while Droloxifene is completely devoid of this activity after at least the same drug exposure of the animals, as shown in Table 4. In view of possible adjuvant or prophylactic long-term administration of anti-estrogens, Droloxifene appears to have considerable advantages over Tamoxifen regarding the risk-benefit assessment.

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News
News
News

In Search of the Perfect SERM: Beyond Tamoxifen And Raloxifene

Never mind this spring's excitement over tamoxifen and raloxifene; they're just forerunners of designer estrogens to come. Second generation compounds are already in clinical trials. And with growing knowledge of how the estrogen receptor works, researchers say that new synthetic estrogens — known as selective estrogen receptor modulators or SERMs — will certainly be entering drug company pipelines over the next decade.

That long view may have been temporarily obscured by headlines emerging last month from the annual meeting of the American Society of Clinical Oncology in Los Angeles, where the two SERMs now on the market, tamoxifen (Nolvadex®) and raloxifene (Evista®), seemed to be competing stars.

Both drugs had slots at the plenary session and at an official ASCO press briefing. Reporting on tamoxifen was D. Lawrence Wickerham, M.D., associate director of the National Surgical Adjuvant Breast and Bowel Project, which conducted the Breast Cancer Prevention Trial. That trial showed that tamoxifen lowered the risk of breast cancer by 45% percent in high-risk women, compared to a placebo (see *News*, May 6, 1998).

A day before the plenary, tamoxifen's maker, Zeneca Pharmaceuticals of Wilmington, Del., had organized a hotel press conference that included high-profile spokespersons such as Harmon J. Eyre, M.D., of the American Cancer Society, and V. Craig Jordan, Ph.D., Northwestern University, Evanston, Ill., whose research led to the development of tamoxifen (see *News*, May 6, 1998). Both emphasized that the tamoxifen findings were based on mature, long-term data. In contrast, they said, much more research was needed on Indianapolis-based Eli Lilly's raloxifene before it could be recommended for the prevention of breast cancer.

For raloxifene, Steven Cummings, M.D., from the University of California at San Francisco, presented early data on the drug's ability to prevent breast cancer. After 2 years of followup among post-menopausal women taking it for osteoporosis, raloxifene appeared to lower the risk of breast cancer by 58% to 66% compared to a placebo. Moreover, it has so far not appeared to increase the risk of endometrial cancer, which is one of the drawbacks of tamoxifen.

Dr. D. L. Wickerham

The two SERMs are destined to meet head-to-head in a second breast cancer prevention trial, which will probably start recruiting patients this fall, according to Wickerham. The Study of Tamoxifen and Raloxifene (STAR) will enroll about 22,000 post-menopausal women who will be randomly assigned to receive either one drug or the other, he added.

While tamoxifen-versus-raloxifene may have been the issue of the week, other ASCO speakers made it clear that eventually there should be more to the SERM story. "The perfect SERM . . . has not yet been developed," said C. Kent Osborne, M.D., of the University of Texas Health Science Center in San Antonio, the discussant for both the tamoxifen and raloxifene presentations. He added that "modern drug discovery techniques offer promise for its synthesis."

The perfect SERM would be a compound that acts as a potent anti-estrogen in the breast and uterus to prevent estrogen-driven cell proliferation and, at the same time, has strong estrogenic effects in bone, the cardiovascular system, and the central nervous system, where hormones can help prevent a variety of post-menopausal conditions.

Second Generation

It is not clear that anything approaching the perfect SERM is now in the pipeline, but a second generation of synthetic estrogens, most of them variations on tamoxifen and raloxifene, are in development.

At Eli Lilly, for instance, drug designers have taken raloxifene's structure as a starting point and are developing a variation that they call SERM III. Their aim, said Dapil Dhingra, M.D., an oncologist and clinical research physician at Lilly, is to optimize the drug's anti-estrogen effects in breast and endometrial tissue.

So far, in preclinical data, the compound does look like a more potent anti-estrogen than raloxifene, Dhingra said. In the clinic, two trials with SERM III are just getting under way; one is a phase II

breast cancer treatment trial and one an early trial of the drug's ability to prevent breast cancer. Lilly is also planning a trial of SERM III to prevent osteoporosis.

Perhaps a Preventive

Another synthetic estrogen in the pipeline is droloxifene, being developed by Pfizer, Inc., New York City. The company originally envisioned droloxifene as a therapeutic drug in breast cancer, but interim data from a phase III trial was discouraging, showing that droloxifene "offered no benefit beyond the current therapy," according to Brian McGlynn, director of corporate media relations.

Pfizer had originally planned to file a New Drug Application for droloxifene with the Food and Drug Administration this year. Now it has dropped that plan, McGlynn said, and decided instead to evaluate droloxifene in the prevention of breast cancer and to accelerate development efforts in osteoporosis, where the data are more promising.

A third SERM in clinical trials is SmithKline Beecham's idoxifene, also designed as a variation on existing SERMs. It is now in a phase III trial for the prevention of osteoporosis and a phase II trial for the treatment of advanced breast cancer. The Philadelphia-based company says idoxifene appears to be estrogenic in bone and anti-estrogenic in the breast, and so far does not increase the risk of endometrial hyperplasia.

If SERM III, droloxifene, idoxifene, and other tamoxifen-like drugs are considered second-generation SERMs, what will the the third generation be like?

"A fundamental change" is needed, said Donald P. McDonnell, Ph.D., a Duke University investigator who has been working on preclinical studies of a SERM called GW5638. (If GW5638 works out in clinical trials, it could offer an option for tamoxifen-resistant cancers, McDonnell said, but it too is basically a variation on current SERMs.)

The next generation of SERMs should be based on new knowledge about estrogen and estrogen receptor biology, McDonnell said, and that is turning out to be much more complex than once thought. For one thing, the different ligands, whether natural estrogens or SERMS, appear to interact with the receptor in different ways.

Knowing the intricacies of those interactions could help in designing new SERMs. For example, recent research using crystallography has clarified the way in which raloxifene and estradiol interact with the estrogen receptor. As Jordan points out in this issue (page 967), that knowledge has provided insight into the mechanisms of anti-estrogenic activity.

Dr. Steven Cummings

But SERM-receptor interactions are not the only events that need to be understood. There are a bevy of other molecules that get involved in a cell's response to estrogen. For instance, scientists recently discovered that there are actually two estrogen receptors, alpha and beta, that occur in different quantities in different cells and tissues. And, as Osborne pointed out, there are at least 20 different receptor interacting proteins that bind to the estrogen receptors and function either as co-activators to enhance estrogen's effect or as co-repressors to inhibit it.

"The estrogen receptor does not work in a vacuum," said McDonnell, speaking at a symposium last March in Chantilly, Va. "It has lots of dancing partners."

And that's not all. There are probably more than 50 transcription activating factors, TAFs, that interact to regulate the effects of estrogen on its target genes. Also at the DNA level, response elements in the promoter regions of the target genes may be involved in the complex process that determines what effect a given SERM will have on tissues.

"Many biologists now feel it is the particular ensemble of ligands, receptors, receptor interacting proteins, and response elements, that determine whether there will be a predominantly agonist or antagonist signal on a given tissue or gene," said Osborne. One of the challenges facing the designers of third-generation SERMs will be defining the workings of these ensembles and developing drugs that target them.

Multiple Options

The third-generation drugs that emerge from this process may be some years away. But the intense interest in SERMs makes it seem certain that eventually clinicians can expect to have an increasing number of options.

"It will be like antibiotics," said Jordan, envisioning a time when there will be many more than two SERMs on the market. In an interview at ASCO, he compared tamoxifen and raloxifene to penicillin, which over the years has been joined by a host of other antibiotics, each with its own indications. One SERM is not necessarily going to replace another, he predicted. Instead, "there will be a menu of options for specific subsets of patients."

— Caroline McNeil

JOURNAL

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Report

Phase III randomized trial of droloxifene and tamoxifen as first-line endocrine treatment of ER/PgR-positive advanced breast cancer

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Key words: advanced breast cancer, droloxifene, positive hormone receptors, randomized trial, tamoxifen

Summary

Purpose: This trial was designed to demonstrate equivalence between droloxifene 40 mg/d and tamoxifen 20 mg/d as first-line treatment in pre- and post-menopausal women with ER+ and/or PgR+ advanced breast cancer based on time to disease progression and tumor response.

Materials and methods: One thousand three hundred fifty four women with measurable disease, previously untreated by hormonal or chemotherapy for advanced or recurrent breast cancer, were enrolled by 179 institutions in 35 countries. Patients were stratified at baseline for menopausal status. Patients receiving adjuvant hormonal therapy within 1 year were excluded. All patients gave written informed consent, were randomized to 40 mg droloxifene or 20 mg tamoxifen daily as single-agent therapy and underwent tumor assessment every 3 months. A central committee reviewed digitized images for all cases of tumor progression or objective response.

Results: The hazard ratio (droloxifene/tamoxifen) for the primary endpoint, time to disease progression, was 1.287 favoring tamoxifen (95% C.I.: 1.114–1.487; $p < .001$). The objective response rate (CR + PR) was 22.4% for droloxifene and 28.6% for tamoxifen ($p = .02$). Tamoxifen was superior to droloxifene overall, among both pre- and postmenopausal patients and among patients ≤65 years; there was no difference among women >65 years. The hazard ratio for all-cause mortality was 0.871 (95% C.I.: 0.672–1.129; $p = .29$), favoring droloxifene but not statistically significant.

Conclusions: Droloxifene was significantly less effective than tamoxifen overall and particularly among women under 65 years. Tamoxifen and droloxifene were both less effective in pre-menopausal women with receptor-positive disease compared to post-menopausal women. Further clinical development of droloxifene was stopped.

Introduction

Droloxifene is a novel selective estrogen receptor modulator (SERM) whose potential as a treatment for breast cancer has been suggested by several non-comparative studies [1–8]. Preclinical studies [9, 10] have shown that droloxifene has a shorter serum half-life and a higher affinity for the estrogen receptor than tamoxifen, an accepted first-line treatment option for many women with hormonally sensitive breast cancer.

*For the Droloxifene 301 Study Group.

Pre-clinically, the two agents differ most with respect to animal carcinogenicity, with tamoxifen shown to cause hepatic tumors in 98% of treated animals while droloxifene- and control-treated animals showed a 1–2% incidence [10]. In humans, of course, it is well known that tamoxifen increases the incidence of endometrial cancer but has no effect on the incidence of hepatic tumors. Nevertheless, these pre-clinical findings suggested that droloxifene might be more useful than tamoxifen for longer-term breast cancer therapy, such as in the adjuvant setting, provided that droloxifene possessed efficacy that was at least equivalent to tamoxifen in patients with advanced disease. A Phase 2 study of 369 women with advanced breast cancer randomized to one of three doses of droloxifene (20, 40 or 100 mg/d) as first-line hormonal therapy gave encouraging results, with CR + PR rates of 30, 47 and 44%, respectively, for the three treatment groups [6]. Droloxifene has also been studied in patients with advanced breast cancer who have been exposed to prior endocrine treatment, including some who were resistant to tamoxifen, with partial response seen in 15% of these patients [11]. The purpose of the present study was to demonstrate equivalence between droloxifene 40 mg daily and tamoxifen 20 mg daily by comparing time to disease progression in a global study intended to reflect the diversity of the patient population actually using first-line hormonal therapy for advanced breast cancer.

Materials and methods

Study design

This was a prospective, randomized, active-control, double-blind, multi-center, parallel-group comparison of droloxifene and tamoxifen, in women with ER+ and/or PgR+ advanced breast cancer. Patients were stratified by menopausal status. The primary endpoint of the trial was time to disease progression, defined as the time from randomization to the first objective finding demonstrating a 25% increase in the size of at least one tumor lesion or the appearance of any new tumor lesion or death due to breast cancer. Overall tumor response was a secondary endpoint, along with response duration and various subset analyses. Time to progression and tumor response were determined for every patient by a central endpoint evaluation committee. Parameters used to pre-define subsets of patients in the prospective statistical analysis plan included age, menopausal status, geography, disease status at baseline, adjuvant therapy, and performance status as listed in Table 4. The independent Data Safety Monitoring Board (DSMB) conducted periodic, planned interim analyses of the data in order to monitor the safety of the trial.

Patient selection

Eligible patients included pre- or post-menopausal women with biopsy-proven breast cancer with distant metastases, locoregional recurrences not suitable for local therapy or inoperable primary tumors. Patients were defined as postmenopausal if menses had ceased for more than 1 year and serum estrogen was below 30 pg/ml, or if the patient had undergone bilateral oophorectomy. Acceptable target lesions were measurable in at least one dimension, at least 1 cm in size and not previously radiated. Lytic bone lesions not visible on plain x-ray were excluded as target lesions, as were any blastic bone lesions or blastic portions of mixed lesions. Patients were excluded if they had received any prior chemo- or hormonal therapy (including oophorectomy) for advanced disease, or adjuvant hormonal therapy within the past year or adjuvant chemotherapy within the past month prior to randomization. Patients were required to have receptor-positive tumors defined as ER+ and/or PgR+ (unknown receptor status for both ER and PgR was not allowed). ECOG performance status of 60% or greater was required. Patients with brain, leptomeningeal or extensive (> 1/3 of the liver) hepatic metastases were excluded, as were patients with hypercalcemia or significant risk for thromboembolic events.

Pretreatment evaluation

Prior to initiating study drug treatment, a complete history, physical exam and tumor assessment were performed, including bone scan, chest x-ray, and abdominal CT (or liver ultrasonography). Any suspicion of bone metastases on the bone scan required a defined set of eight skeletal plain films for confirmation. Representative tumor lesions were identified for each patient. Tumor response or progression would be determined based upon changes in these target lesions. Any new lesion was deemed disease progression regardless of changes in target lesions.

Treatment plan

Each patient received, by random assignment, either (a) 40 mg/day droloxifene (Pfizer Central Research,

Groton, CT) + placebo tamoxifen or (b) 20 mg/day tamoxifen (Tamoxpuren®, Klinge Pharma, Munich, Germany) + placebo droloxifene.

Follow-up tumor assessments

After randomization, patients returned to clinic every 3 months. At each visit, physical examination, chemistry/hematology, chest x-ray, and measurement of target lesions were performed for all patients. Abdominal CT (or hepatic sonography) and/or bone scan with skeletal x-ray series were performed every 3 months for those patients with relevant target lesions at baseline. Patients with no target lesions in either bone or liver received abdominal CT and bone scan at 6 month intervals and at the end of the study.

Response evaluation

Target lesion measurements were recorded in a log every 3 months. All physical examination measurements were checked for errors between the medical record and the case report forms by monitors who visited each study center at least every 4–8 weeks. All x-ray and scan images of target lesions, or new lesions, were first evaluated by each investigator in order to determine the clinical plan for the patient. These films were then sent to a central imaging facility where each x-ray or scan was digitized for electronic review by an Endpoint Classification Committee (ECC) consisting of experienced investigators and radiologists from North America and Western Europe. Electronic images were viewed on a bank of four ultra-high resolution monitors using software that allowed contrast adjustment to optimize image readability of x-rays and scans. The reviewers were blinded to treatment arm. Tumor response was evaluated according to WHO criteria [12], with additional requirements that (i) only x-ray, CT or MRI (not radionuclide bone scan) were used to determine response or progression in bone, and (ii) blastic bone lesions, or the blastic portion of mixed lytic/blastic bone lesions, were not considered for tumor response evaluation. The decision of the committee regarding objective tumor response and the date(s) related to that response was final as concerned the study analyses. This committee reviewed every case in which the investigator found CR, PR or disease progression, a death or a premature termination. At the close of the trial, all patients still receiving their assigned study medication underwent a termination visit and complete tumor assessment. Only those active patients whose disease status was unchanged at the time the trial closed, according to the investigator's review of the case, were accepted as 'no change' in the trial database without committee review.

Ethics

The ethical committee at each participating institution reviewed and approved the protocol and the informed consent document. Each patient gave written informed consent that met the requirements of FDA GCP regulations and the Declaration of Helsinki (as amended 1975 and 1983), in addition to all local regulations in each country as required.

Statistical methodology

The statistical plan predicted that the study would need to enroll 1375 patients in order to observe 900 events (disease progressions) within 2 years. Patients were assigned to study treatment by a computer-generated randomization list after stratification by menopausal status. The study was designed as a non-inferiority trial employing the technique of repeated confidence intervals [22]. The trial was planned to continue until 900 events had occurred in order to allow a determination that the relative efficacy of droloxifene was at least 80% that of tamoxifen as measured by the hazard ratio for time to disease progression. The operating characteristics of the statistical inference were such that the power was 90% to declare the non-inferiority of droloxifene relative to tamoxifen if the true times to disease progression for the two drugs were not different and approximately 900 events had been observed. The statistical plan allowed stopping the trial before 900 events for a statistically significant difference in efficacy, but required observation of 900 events in order to declare non-inferiority. Interim analyses were scheduled to occur with every 150 additional events and these results were provided only to the independent DSMB in order for them to review the progress and safety of the trial. However, the identity of each treatment arm remained coded until after the DSMB had made the decision to end the trial. The project medical and administrative staff, along with investigators and other study personnel, were unaware of any interim results.

Hazard ratios are estimated from univariate proportional hazards regression (Cox) models with treatment as the sole predictor. Lifetime analyses are based on the product-limit method of Kaplan and Meier. Confidence intervals and p-values reported herein are nominal, that is, unadjusted for multiple comparisons

Table 1. Geographic regions (randomized patients)

Africa/Mid-East (201 patients)	Egypt	South Africa	Israel
Asia (337 patients)	P. R. China India	Hong Kong	Taiwan
Eastern Europe (210 patients)	Russia Hungary Czech Republic	Serbia Latvia Slovakia	Belarus Poland
Latin America (91 patients)	Mexico Brazil	Argentina Chile	Costa Rica Uruguay
North America (252 patients)	Canada	United States	Puerto Rico
Western Europe (263 patients)	France Germany Netherlands Austria	United Kingdom Turkey Greece Spain	Sweden Belgium Norway Italy

or interim analyses. The log-rank test was used for comparisons of time to event distributions. The chi-square test was used for comparisons of response rates. All of the subgroup analyses shown in Tables 4 and 5 were included in the prospective statistical analysis plan and were hypothesis-testing analyses.

Results

Patient characteristics

A total of 1966 women with advanced breast cancer were screened for the trial and 1354 women were randomized between June, 1995 and December, 1997 at 179 study centers in 35 countries and territories (Table 1).

The pretreatment characteristics of the patients are shown in Table 2 according to treatment group. The two treatment groups showed no significant differences with respect to the parameters in Table 2, with the exceptions that the patients in the droloxifene group were somewhat more likely ($p < .05$; chi-square) to have four or more tumor lesions at baseline and to have received prior adjuvant hormonal or radiation therapy.

The mean duration of therapy was 196 days (range: 8–920 days) in the droloxifene group and 218 days in the tamoxifen group (range: 6–969 days). A total of 69 patients discontinued the study before disease progression occurred. Reasons for early termination are shown in Table 3. Under intention-to-treat principles, all randomized patients were included in the analyses of safety and efficacy.

Central review of endpoints

The ECC (ECC; A. Buzdar, Chairman) centrally reviewed 1026 patients for tumor response out of 1354 enrolled patients. A total of 328/1354 cases were reported by the investigator to be 'no change' at the time of the study data cut-off (February 1998); these cases were not submitted for review by the ECC. Every case involving disease progression or complete or partial response (CR or PR), as judged by the investigator, was reviewed centrally. The committee determined the nature of the response and the date of response or progression for the purposes of the analysis. The ECC was unable to adjudicate 36/1026 cases (3.5%) due to insufficient data.

Disease progression

Time to disease progression was the primary endpoint of the study. Figure 1 shows the time-course of disease progression for all randomized patients by treatment group. More than half of the patients (744/1354) experienced disease progression during the

Table 2. Pretreatment characteristics of randomized patients by treatment group and region (no. patients unless otherwise indicated)

		Droloxifene	Tamoxifen	Europe West	Europe East	Africa/M.E.	Asia	Latin America	North America
No. of patients randomized		681	673	263	210	201	337	91	252
Age	Under 45 years	115	96	7%	4%	14%	32%	13%	14%
	45–64 years	347	330	34%	56%	53%	57%	60%	42%
	65+ years	219	247	59%	40%	33%	11%	27%	44%
Menopausal status	Premenopausal	138	123						
	Postmenopausal	542	550						
Mean weight	(kilograms)	66.0	65.2						
Karnofsky score	80–100%	577	573	89%	87%	95%	80%	85%	77%
	60–70%	103	99	11%	13%	5%	19%	15%	23%
	<60%	1	1	0%	0%	0%	1%	0%	0%
Race	White	433	425						
	Asian	175	173						
	Black	26	25						
	Other	47	48						
	Not provided	0	2						
Disease at baseline	Recurrent disease, locoregional only	69	78	10%	6%	15%	11%	15%	11%
	Recurrent disease, distant metastases	297	270	48%	19%	24%	47%	38%	63%

Table 2. (continued)

		Droloxifene	Tamoxifen	Europe West	Europe East	Africa/M.E.	Asia	Latin America	North America
	Primary diagnosis, locally advanced only	95	110	24%	27%	19%	7%	21%	1%
	Primary diagnosis, distant metastases	186	182	16%	46%	33%	27%	24%	20%
	Primary diagnosis, not classified	34	33	2%	2%	8%	7%	2%	5%
Disease-free interval	0–24 months	452	445	59%	82%	79%	74%	65%	40%
	>24 months	218	219	40%	18%	21%	25%	34%	56%
	Not available	11	9	1%	0%	0%	1%	1%	4%
Estrogen receptor	Positive	610	622	94%	89%	95%	85%	95%	94%
	Negative or unknown*	71	51	6%	11%	5%	15%	5%	6%
Progesterone receptor	Positive	457	462	76%	53%	55%	77%	41%	80%
	Negative or unknown*	224	211	24%	47%	45%	23%	59%	20%
Distant metastases	Bone mets only	79	88	14%	7%	5%	8%	21%	23%
	Bone and non-bone mets	117	99	9%	12%	14%	27%	3%	18%
	Bone mets absent	457	465	72%	80%	78%	62%	74%	53%
	Not classified	28	21	5%	1%	3%	3%	2%	6%
	Liver metastases present	85	75	9%	6%	10%	18%	4%	16%
	Liver metastases absent	576	584	87%	94%	87%	81%	94%	80%
	Not classified	20	14	4%	0%	3%	1%	2%	4%
No. of tumor lesions	1–3	460	498	86%	78%	73%	53%	82%	67%
	4 or more	201	161	10%	22%	25%	46%	16%	29%
	Not classified	20	14	4%	0%	2%	1%	2%	4%

Table 2. (continued)

		Droloxifene	Tamoxifen	Europe West	Europe East	Africa/M.E.	Asia	Latin America	North America
Adjuvant hormonal therapy**	Prior adjuvant hormonal therapy	79	61	32%	17%	14%	12%	18%	21%
	No adjuvant hormonal therapy	287	287	68%	83%	86%	88%	82%	79%
Adjuvant chemotherapy**	Prior adjuvant chemotherapy	185	184	32%	51%	34%	65%	55%	47%
	No previous chemotherapy	181	164	68%	49%	66%	35%	45%	53%
Previous radiation	Yes	206	170	19%	20%	16%	27%	38%	36%
	No	475	503	81%	80%	84%	73%	62%	64%
Surgery on primary tumor	Mastectomy (all types)	288	272	39%	25%	30%	50%	40%	56%
	Segmental mastectomy/tumorectomy	72	67	17%	2%	5%	4%	15%	20%
	No surgery (biopsy only)	272	295	33%	69%	57%	40%	37%	17%
	Other surgery/unknown	49	39	11%	4%	8%	6%	8%	7%

*Each patient in this category was known to be 'positive' for the other receptor, either ER or PgR.
**Among patients with recurrent disease.

Table 3. Reasons for early termination

No. (%) of Patients	Droloxifene	Tamoxifen
Randomized	681	673
Discontinuations	37 (5.4%)	32 (4.8%)
Protocol violation	3	1
Lost to follow-up	10	5
Withdrew consent	9	8
Adverse event	9	14
Other	6	4

course of the study. The hazard ratio for disease progression (droloxifene: tamoxifen) was 1.287 (95% C.I.: 1.114–1.487; $p < .001$), indicating a significantly lower risk for disease progression in the tamoxifen group. The median time to progression, for all patients, was 228 days for tamoxifen and 183 days for droloxifene. Early disease progression (defined as progression $\leq$3 months from randomization) occurred in 234/613 (38%) droloxifene patients who had at least 3 months of follow-up and in 186/623 (30%) of tamoxifen patients with similar follow-up. The incidence of disease progression and the hazard ratios for the predefined subpopulations are shown in Table 4. Tamoxifen showed a lower risk for disease progression than droloxifene among both pre- and post-menopausal patients. The hazard ratios for these two strata were not statistically different, although the superiority of tamoxifen was somewhat more pronounced among the pre-menopausal stratum. One patient could not be classified with respect to menopausal status and was censored from that subgroup analysis. A multivariate analysis of the predefined prognostic factors showed that the statistical superiority of tamoxifen was still evident after adjustment of these factors.

Survival

A total of 230/1354 patients died while on the study. The endpoint committee reviewed each death. It was determined that 10/230 deaths were due to causes other than the disease under study, however, all deaths were included in the analysis of overall survival. Survival is depicted by treatment group in Figure 2 for all patients. The hazard ratio for all-cause mortality was 0.871, favoring droloxifene but not statistically significant (95% C.I.: 0.672–1.129). Differences in survival favoring droloxifene were found in three subgroup analyses: patients with a disease-free interval >24 months (hazard ratio = 0.528, 95% C.I.: 0.317–0.879); patients who received previous adjuvant hormonal therapy (hazard ratio = 0.424, 95% C.I.: 0.193–0.932); and patients who received previous adjuvant chemotherapy (hazard ratio = 0.532, 95% C.I.: 0.319–0.887). These results are unadjusted for multiple comparisons and are considered hypothesis generating only. No subgroup analysis of survival showed a significant finding in favor of tamoxifen.

Objective tumor response rate and duration

Overall, 121/681 (18%) droloxifene patients and 155/673 (23%) tamoxifen patients had a confirmed response to treatment (PR or CR). However, these denominators include a number of patients who were randomized less than 6 months before the study was concluded and had not yet experienced a prespecified endpoint (CR, PR or disease progression). Therefore, best response data are presented for the 541 droloxifene patients and 542 tamoxifen patients who (a) had a documented PR or CR or (b) had a documented disease progression or (c) in the absence of CR, PR or disease progression, had been observed for at least 6 months. The best responses for this group of 1083 patients are shown in Table 5 as a function of treatment and disease characteristics. The tumor response rates for both droloxifene and tamoxifen were markedly lower among pre-menopausal patients (5.2 and 15.5%, respectively) as compared to post-menopausal patients (27.1 and 31.7%, respectively). The remaining 271 patients had enrolled within the final 6 months of the study. These patients all had stable disease with less than 6 months of observation and were censored from this analysis; because the study ended, no additional follow-up of these patients was available.

Among the 22/261 pre-menopausal and 254/1039 post-menopausal patients who did show a response (CR or PR) during the study, the overall hazard ratio (droloxifene:tamoxifen) for time to response was 1.053 (95% C.I.: 0.829–1.337; $p = .67$) suggesting no difference between the treatments. The median time to response was approximately 90 days in each treatment group and was strongly influenced by the protocol's follow-up visit schedule (visits every 3 months). However, only 25% of patients who would eventually respond did so after 100 days, and less than 10% after 180 days. The median duration of response was 452 days for the tamoxifen group and 555 days for the droloxifene group (NS).

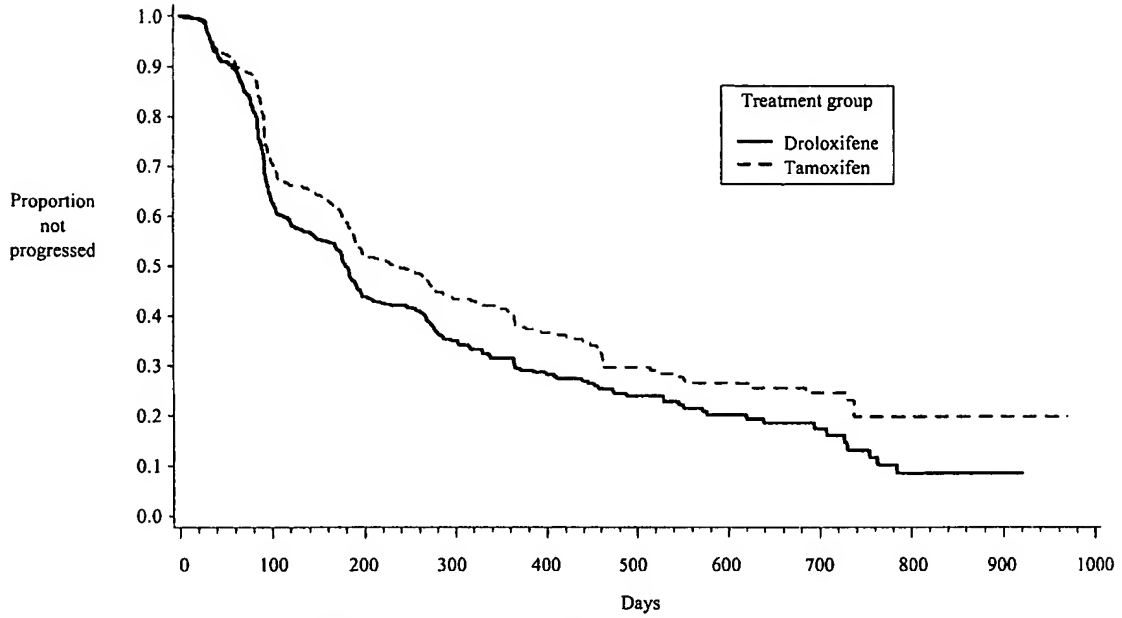

Figure 1. Kaplan–Meier curve – proportion not progressed by treatment group (product limit estimator).

Safety and tolerability

Approximately 70% of each treatment group reported one or more treatment-emergent adverse events during the trial. The incidence rates for these events are shown in Table 6. Serious adverse events, defined as hospitalization, cancer, other life-threatening conditions and any death by current FDA Good Clinical Practice regulations, occurred in 110 patients in each group and included hypercalcemia, thromboembolic events and death due to breast cancer.

Decision to stop trial

Two interim analyses were conducted. A provisional decision to end the study was made by the DSMB after the second interim analysis (307 events) based on conditional power calculations that suggested that the test drug was likely to be declared inferior to the control agent. In particular, the superior efficacy of the control in the younger, pre-menopausal population was the underlying reason that the DSMB decided to stop the study early. Further analyses were conducted in order to confirm that no subgroup or anomaly could account for the emerging statistical superiority of the standard treatment. The trends seen at the second interim analysis were born out in the final results. As a result of these findings the trial was stopped after 744 events had taken place among 1354 randomized patients. It was not necessary to proceed to 900 events, as originally planned, in order to draw a conclusion.

Discussion

The results of this study show that droloxifene has activity in hormonally sensitive advanced breast cancer when given as first-line therapy, but based on the time to disease progression and tumor response results, its overall efficacy is inferior to that of tamoxifen. The tumor response rate for droloxifene was clearly less than that for tamoxifen among women under 65 years (15% v.s. 23%, respectively), whereas the two drugs showed the same efficacy (38%) among patients 65 or over.

The reason both drugs were significantly less effective among younger or pre-menopausal patients, despite any baseline differences, cannot be definitively determined from the results of this study. Overexpression of the HER-2/c-neu/c-erbB-2 protooncogene has been shown to predict lower response to hormone therapy and is more common among premenopausal women [19, 20]. While erbB-2 expression was not determined in this trial, a separate study of droloxifene in 94 advanced breast cancer patients showed that response rate was 9% among women with high pretreatment circulating levels of the extracellular

Table 4. Time to disease progression: hazard ratios (droloxifene:tamoxifen) (95% C.I.)*

Cohort	No. disease progressions/ No. randomized (%PD)	Hazard ratio (95% C.I.)
All Patients	744/1354 (54.9)	1.287 (1.114–1.487)
Age 65 or more	197/466 (42.3)	1.045 (0.790–1.383)
Age 45–64	393/677 (58.1)	1.391 (1.140–1.698)
Age 44 or less	154/211 (73.0)	1.339 (0.972–1.845)
Premenopausal	173/261 (66.3)	1.476 (1.091–1.997)
Postmenopausal	570/1092 (52.2)	1.224 (1.039–1.443)
Western Europe	117/263 (44.5)	1.350 (0.937–1.944)
Eastern Europe	100/210 (47.6)	1.307 (0.881–1.940)
Africa/Middle East	112/201 (55.7)	1.019 (0.703–1.477)
Asia	217/337 (64.4)	1.331 (1.019–1.738)
Latin America	46/91 (50.5)	1.870 (1.036–3.374)
North America	152/252 (60.3)	1.141 (1.026–1.949)
Distant metastases present	539/939 (57.4)	1.337 (1.128–1.584)
Distant metastases absent	167/355 (47.0)	1.201 (0.884–1.630)
Primary breast cancer	336/640 (52.5)	1.297 (1.046–1.607)
Recurrent breast cancer	408/714 (57.1)	1.284 (1.057–1.560)
Bone metastases only	90/167 (53.9)	1.183 (0.781–1.791)
Not bone metastases only	633/1138 (55.6)	1.308 (1.119–1.530)
Liver metastases present	111/160 (69.4)	1.132 (0.775–1.655)
No liver metastases	620/1160 (53.4)	1.312 (1.121–1.536)
Four or more tumor lesions	258/362 (71.3)	1.305 (1.018–1.673)
Three or fewer tumor lesions	473/958 (49.4)	1.210 (1.010–1.450)
Disease-free interval ≤24 mos.	494/897 (55.1)	1.378 (1.086–1.759)
Disease-free interval >24 mos.	239/437 (54.7)	1.120 (0.869–1.444)
Prev. adjuv. hormonal**	86/140 (61.4)	1.182 (0.763–1.832)
No prev. adjuv. hormonal**	322/574 (56.1)	1.277 (1.026–1.589)
Prev. adjuv. chemo**	220/345 (63.8)	1.558 (1.192–2.037)
No prev. adjuv. chemo**	188/369 (50.9)	1.045 (0.785–1.392)
Karnofsky 80–100%	611/1150 (53.1)	1.270 (1.083–1.489)
Karnofsky 60–70%	131/202 (64.9)	1.394 (0.987–1.968)

*In some cases the total number of patients shown in complementary categories may be less than the total number of randomized patients ($N = 1354$) due to unclassified patients.
**Only patients with recurrent disease ($n = 714$) are included in these analyses.

domain of the HER-2/c-neu protein, but was 56% among women with low circulating levels [21]. However, the decrement in efficacy experienced by premenopausal women in the present study is even more dramatic for droloxifene than for tamoxifen. It may be that the shorter serum half-life of droloxifene (1 day) as compared to tamoxifen (1 week), with the resulting fluctuations in serum droloxifene levels, could be an important factor. The kinetics of droloxifene in tumor tissue have not been examined, although this has been done for tamoxifen [13]. It is plausible that the active tamoxifen metabolite (4-OH-tamoxifen) is able to compete more effectively than droloxifene for the estrogen receptor in the face of endogenous estrogen, as would be the case in the pre-menopausal patient. One could speculate that a higher dose of droloxifene could have overcome this disadvantage, and there is some evidence for this. A series of investigations carried out to study the effects of tamoxifen and droloxifene on serum levels of sex hormones, IGF-1 and associated binding proteins in breast cancer patients [14–18] has shown that these effects are dose related for droloxifene between 40 mg/d, the dose in the present study, and 100 mg/d. In addition, other data obtained in

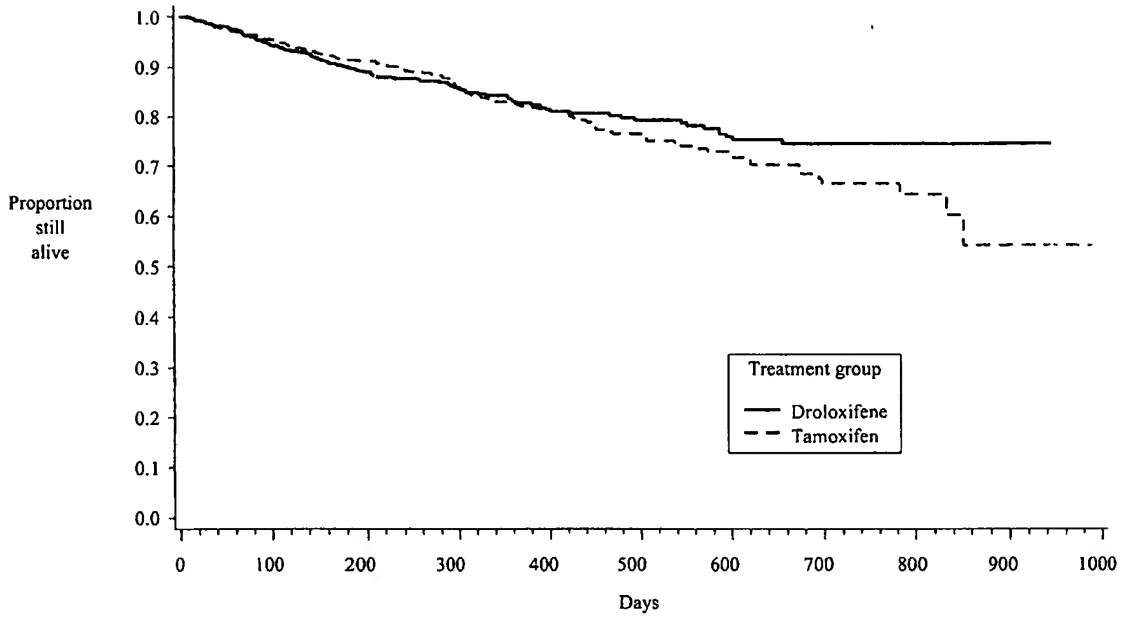

Figure 2. Kaplan–Meier curve – proportion still alive by treatment group (product limit estimator).

normal postmenopausal women (unpublished observations) demonstrate that the efficacy of droloxifene for other clinical endpoints (bone density, lipid lowering) does continue to increase substantially at doses beyond 40 mg. These effects are primarily estrogen-agonist effects. While no Phase II breast cancer trials with droloxifene have provided any convincing evidence that this is the case for anti-estrogen effects in breast tissue [1–8], these earlier trials, all much smaller than the present study, had little statistical power to discriminate between two active doses. Moreover, premenopausal women were not well represented in the Phase II breast cancer trials. Finally, given that these results show tamoxifen efficacy to be much lower in younger patients, one could speculate that a tamoxifen dose higher than the standard 20 mg/day could increase efficacy in younger or premenopausal patients treated with tamoxifen.

The wide geographic reach of this study provides some interesting insight into the nature of a global patient population, and their response to therapy. However, it should be understood that the statistics seen here do not necessarily represent the epidemiology of breast cancer in these countries, but rather reflect the patients available and willing to participate in a research study. As such, they may be influenced by factors other than incidence, for example, cultural factors. Our findings do suggest that advanced disease is less commonly found at initial diagnosis in the West and that increased measures aimed at early detection of breast cancer could benefit additional patients in many areas of the world. Given that all patients in this study were known to be ER+/PgR+, the more frequent use of adjuvant chemotherapy in some regions, with less use of adjuvant tamoxifen, may be a reflection of lower utilization of receptor testing at initial staging, with the result that some study patients with recurrent disease were identified as being ER+/PgR+ only at the time they were screened for this study. Availability of medications may also be a factor. Apparent differences in efficacy between regions are likely due to differences in disease characteristics at baseline (Table 4), with more extensive disease, younger age and somewhat poorer physical condition being more frequently noted in the statistics for developing countries. The relative efficacy of the two drugs does not appear to differ in any fundamental way when examined regionally.

Patients receiving droloxifene showed a non-significant trend toward improved overall survival compared to those on tamoxifen. There were statistically significant differences in survival in three subgroups: patients who received previous adjuvant hormonal therapy, patients who received previous

Table 5. Tumor response by treatment and disease characteristics

	Droloxifene		Tamoxifen		
	n	%	*n*	%	*p*
Randomized	681		673		
Insufficient follow-up	140		131		
Overall (evaluated patients)	541		542		
CR	20	3.7	27	5.0	
PR	101	18.7	128	23.6	
CR + PR	121	22.4	155	28.6	.02
NC (6 months)	111	20.5	124	22.9	
Age < 65 years	171		201		
CR	12	7.0	12	6.0	
PR	53	31.0	65	32.3	
CR + PR	65	38.0	77	38.3	.95
NC (6 months)	45	26.3	53	26.4	
Age < 65 years	370		341		
CR	8	2.2	15	4.4	
PR	48	13.0	63	18.5	
CR + PR	56	15.1	78	22.9	.01
NC (6 months)	18	4.9	21	6.2	
Postmenopausal*	425		439		
CR	18	4.2	23	5.2	
PR	97	22.8	116	26.4	
CR + PR	115	27.1	139	31.7	.14
NC (6 months)	93	21.9	103	23.5	
Premenopausal*	115		103		
CR	2	1.7	4	3.9	
PR	4	3.5	12	11.7	
CR + PR	6	5.2	16	15.5	.01
NC (6 months)	18	15.7	21	20.4	
Prior adjuvant hormonal therapy	63		47		
CR	0	0	1	2.1	
PR	10	15.9	6	12.8	
CR + PR	10	15.9	7	14.9	.89
NC (6 months)	14	22.2	15	31.9	
No prior adjuvant hormonal therapy	287		287		
CR	13	4.5	16	5.6	
PR	27	9.4	46	16.0	
CR + PR	40	13.9	62	21.6	.02
NC (6 months)	42	14.6	47	16.4	
US, Canada, West Europe	216		210		
CR	14	6.5	10	4.8	
PR	43	19.9	54	25.7	
CR + PR	57	26.4	64	30.5	.35
NC (6 months)	51	23.6	62	29.5	
Latin America Asia, Africa, East Europe	325		332		
CR	6	1.8	17	5.1	

Table 5. (continued)

	Droloxifene		Tamoxifen		
	n	%	*n*	%	*p*
PR	58	17.8	74	22.3	
CR + PR	64	19.7	91	27.4	.02
NC (6 months)	60	18.5	62	18.7	
Recurrent disease	288		283		
CR	13	4.5	17	6.0	
PR	37	12.8	52	18.4	
CR + PR	50	17.4	69	24.4	.04
NC (6 months)	56	19.4	62	21.9	
New diagnosis of BC	253		259		
CR	7	2.8	10	3.9	
PR	64	25.3	76	29.3	
CR + PR	71	28.1	86	33.2	.21
NC (6 months)	55	21.7	62	23.9	
Locoregional disease only*	132		156		
CR	7	5.3	9	5.8	
PR	47	35.6	60	38.5	
CR + PR	54	40.9	69	44.2	.57
NC (6 months)	27	20.5	32	20.5	
Distant metastases*	383		361		
CR	12	3.1	16	4.4	
PR	50	13.1	62	17.2	
CR + PR	62	16.2	78	21.6	.06
NC (6 months)	77	20.1	91	25.2	
Bone metastases only	63		72		
CR	1	1.6	2	2.8	
PR	5	7.9	12	16.7	
CR + PR	6	9.5	14	19.4	.11
NC (6 months)	20	31.7	20	27.8	
Liver metastases	66		58		
CR	2	3.0	1	1.7	
PR	2	3.0	4	6.9	
CR + PR	4	6.1	5	8.6	.58
NC (6 months)	7	10.6	8	13.8	
Four or more lesions at baseline	174		132		
CR	2	1.1	2	1.5	
PR	17	9.8	23	17.4	
CR + PR	19	10.9	25	18.9	.04
NC (6 months)	24	13.8	19	14.4	
Karnofsky score <80	103		99		
CR	0	0	3	3.0	
PR	8	7.8	8	8.1	
CR + PR	8	7.8	11	1.1	.42
NC (6 months)	12	11.7	14	14.1	

*Presence or absence of distant metastases could not be confirmed for 51 patients (26 droloxifene; 25 tamoxifen). One patient (droloxifene) unclassified for menopausal status.

Table 6. Treatment-emergent adverse events (% incidence)

	Droloxifene 40 mg	Tamoxifen 20 mg
Randomized patients	681	673
Adverse events (in percentage)		
Headache	6	7
Hot flushes	13	14
Nausea	11	11
Back pain	8	8
Vomiting	7	7
Dyspnea	8	7
Hypercalcemia	1	1
Pulmonary embolism	0.1	0.4
Other thromboembolic events	1	1
Vaginal bleeding	0.4	1.5
Endometrial hyperplasia	0	0.1
Endometrial carcinoma	0	0
Retinal disorders	0	0.4
Cataract	0.3	0
Any serious adverse event (SAE)	16	16

adjuvant chemotherapy, and patients with a disease-free interval greater than 24 months. These subgroup results all favored droloxifene and are considered to be hypothesis generating.

The overall conclusions of the study are (i) that droloxifene 40 mg/d is less effective than tamoxifen 20 mg/d and (ii) that either drug is markedly less effective in pre-menopausal patients compared to postmenopausal. In light of these results, no further development of the droloxifene will be undertaken.

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Biological Significance of Interventions That Change Breast Density

Rowan T. Chlebowski, Anne McTiernan

Mammography provides an image, albeit imperfect, of what is inside the breast. Women with high levels of mammographic density, which reflects the relative proportion of glandular and stromal tissues to fatty tissue in the breast, have been consistently shown in observational studies to have a high risk of developing breast cancer *(1–3)*. In this issue of the Journal, Greendale and colleagues *(4)* report the effect of 12 months of postmenopausal hormone therapy on mammographic density among women enrolled in the randomized clinical Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial, which tested the effects of several regimens of estrogen plus progestin, estrogen alone as conjugated equine estrogens, and placebo.

The current report builds upon a previous observation in the same PEPI study population *(5)* by using a computer-assisted technique to provide a continuous measure of the proportion of the breast area that is comprised of dense tissue. The authors' comparison to a categorical evaluation of density using the four-level Breast Imaging Reporting and Data System (BI-RADS) density grades *(6)* is informative, and highlights the need for standardized measurement criteria in this area of research. However, in the current report, it is not clear why the authors provide an adjusted analysis rather than an intent-to-treat comparison between the intervention arms and placebo arm of the study. This decision suggests that there may have been imbalance between the study arms with respect to mammographic density at study baseline. In any event, the authors observed a statistically significant increase in breast density among women who received the estrogen plus progestin combinations but not for those who received estrogen alone, which is consistent with prior reports *(5,7–10)*, at least for continuous combinations of these hormones *(11)*. The effects of the hormone preparations, however, were relatively small in that there was a less than 5% absolute mean change in the proportion of breast area made up of dense tissue. The authors themselves raise the most critical question—what is the link between such changes in breast density in women receiving postmenopausal hormone therapy and subsequent changes in breast cancer risk?

Recently, a substantial genetic component has been associated with the underlying differences in breast density among women *(12)*. In addition, high mammographic density in greater than 75% of the breast area has been associated with an increased incidence of proliferative histologies and/or noninvasive cancers in the breast *(13)*. Such observations perhaps explain much of the relationship between substantial, long-standing high breast density and breast cancer risk, but they leave a different question unanswered: Does a relatively short-term, modest change in breast density that is associated with an intervention carry the same breast cancer risk implications as does underlying differences in breast density? Moreover, does breast density change represent a modifiable risk factor that might be useful in identifying intervention strategies?

The available literature on intervention studies with breast density endpoints can be viewed as making a series of predictions; many of these predictions are currently under evaluation in full-scale randomized clinical trials of breast cancer risk reduction. These predictions and the status of potentially confirmatory trials are outlined below according to the type of intervention tested. Estrogen plus progestin has been shown to increase breast density, whereas estrogen alone has had a limited or no measurable effect on this endpoint *(5,7,8)*. Tamoxifen has fairly consistently decreased breast density *(14–17)*, whereas the effects of raloxifene on breast density have been mixed *(18,19)*. Finally, dietary fat intake reduction also reduced breast density in a prospective trial *(20)*.

Information will be available soon from randomized clinical trials that are designed to determine the effects of such interventions on breast cancer outcome. Indirectly, these trials will provide information on the value of short-term changes in breast density in predicting an intervention effect on breast cancer risk. One such trial, within the Women's Health Initiative (WHI) with 16 608 postmenopausal women participants, has reported an increase in the number of breast cancers after approximately 5 years of combined conjugated equine estrogens plus medroxyprogesterone acetate use *(21)*. Another WHI randomized trial that is evaluating estrogen alone for women with prior hysterectomy continues with active Data Safety Monitoring Board oversight of 10 739 randomized women. The National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 trial *(22)* and several European trials *(23–25)* have followed more than 27 000 women who were randomly assigned to receive tamoxifen or placebo for primary breast cancer risk reduction. Taken together, the results of these trials demonstrate an overwhelming proof of principle regarding a favorable tamoxifen effect on breast cancer risk, albeit with a clinical utility that is limited by endometrial and vascular side effects *(26)*. The Raloxifene for Use in The Heart (RUTH) trial has randomly assigned 10 211 women who are at a high risk of heart disease to raloxifene or placebo, includes a baseline assessment of breast cancer risk, and has added breast cancer risk as an additional primary endpoint *(27)*. The NSABP P-2 trial, which is comparing raloxifene with tamoxifen, is in the process of successfully completing accrual as scheduled and currently has more than 14 000 participants *(28)*. Dietary fat intake reduction is undergoing randomized clinical trial testing in a population of more than 48 000 postmenopausal women in the WHI Dietary Modification trial for primary prevention of breast cancer *(29)*. In addition, accrual has been completed for two randomized secondary prevention (ad-

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juvant) trials of dietary change, with over 5000 breast cancer patients participating *(30–32)*.

In the WHI, an ancillary study is evaluating relationships among breast density change, study arm, and clinical outcome. Similar or more concerted efforts incorporating breast density change in other ongoing randomized clinical trials targeting breast cancer risk could help to directly evaluate breast density as a modifiable risk factor for breast cancer. It will be of equal importance to test interventions to reduce mammographic density in premenopausal women *(33)*, a group that has a high prevalence of increased mammographic density.

In summary, mammographic density is a useful marker of increased risk for breast cancer and of decreased sensitivity of mammographic diagnoses, and may be a valid marker of the effect of interventions that increase or decrease breast cancer risk. Only time, and ongoing randomized trials, will tell.

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Note

Editor's note: R. Chlebowski is a consultant for Astra-Zeneca (Wilmington, DE), the manufacturer of tamoxifen, and A. McTiernan is a principal investigator of a pharmaceutical company (Besins International U.S., Inc.)-sponsored clinical trial of the effects of a tamoxifen derivative on mammographic density.

Digitized Mammography: a Clinical Trial of Postmenopausal Women Randomly Assigned to Receive Raloxifene, Estrogen, or Placebo

Matthew Freedman, Javier San Martin, John O'Gorman, Stephen Eckert, Marc E. Lippman, Shih-Chung B. Lo, Erin L. Walls, Jianchao Zeng

Background: **High mammographic density is associated with increased breast cancer risk. Previous studies have shown that estrogens increase breast density on mammograms, but the effect on mammographic density of selective estrogen receptor modulators, such as raloxifene, is unknown. We assessed changes in mammographic density among women receiving placebo, raloxifene, or conjugated equine estrogens in an osteoporosis prevention trial.** *Methods:* **In a 5-year multicenter, double-blind, randomized, placebo-controlled osteoporosis prevention trial, healthy postmenopausal women who had undergone hysterectomy less than 15 years before the study and had no history of breast cancer received placebo, raloxifene (at one of two doses), or conjugated estrogens (ERT). Women from English-speaking investigative sites who had baseline and 2-year craniocaudal mammograms with comparable positioning (n = 168) were eligible for this analysis. Changes in mammographic density were determined by digital scanning and computer-assisted segmentation of mammograms and were analyzed with the use of analysis of variance. All statistical tests were two-sided.** *Results:* **Among the four treatment groups after 2 years on study, the mean breast density (craniocaudal view) was statistically significantly greater in the ERT group than it was in the other three groups ($P<.01$ for all three comparisons). Within treatment groups, the mean breast density from baseline to 2 years decreased statistically significantly in women receiving the placebo or either the higher or lower raloxifene dose ($P = .003$, $P = .002$, and $P<.001$, respectively) and showed a nonstatistically significant increase in women receiving ERT.** *Conclusions:* **In an osteoporosis prevention trial, raloxifene did not increase breast density after 2 years of treatment. Raloxifene administration should not interfere with, and could even enhance, mammographic detection of new breast cancers. [J Natl Cancer Inst 2001;93: 51–6]**

When mammographic screening programs were found in the early 1980s to decrease the mortality rate due to breast cancer *(1)*, the use of mammograms became standard clinical practice. In addition to detecting existing breast cancer, mammography can also be useful for assessing breast density, which was first proposed as a risk factor for breast cancer in 1976 *(2)*. High breast density is a strong predictor of breast cancer, behind only age and family history of breast cancer *(3)*, and is directly correlated with a fourfold to sixfold increase in breast cancer risk *(2,4–9)*. Identifying women at high risk for breast cancer is increasingly important as preventive therapies are being studied and becoming available for that population *(10,11)*.

High mammographic density can obscure subtle breast abnormalities, making it not only more difficult to diagnose small-volume breast cancer but also more likely for a woman to have a false-positive mammogram reading *(12)*. Approximately 50% of breast cancers are detected by mammography as masses *(13)*. Such masses are more difficult to detect and to classify than are microcalcifications because surrounding radiographically dense epithelial or glandular tissue can obscure masses, especially in women with radiologically dense breasts *(14)*.

Breast density, as measured by mammography, is determined by the relative amounts of fat and fibroglandular tissue present. Fat is radiolucent and appears dark on radiography. In contrast, fibroglandular tissue, which contains a mixture of fibrous connective tissue (stroma) and glandular tissue (epithelial cells), is more dense and appears white on mammograms. The Wolfe classification *(15)* uses visual assessments of the relative percentage of fat and fibroglandular tissue to determine breast density. This method identifies four parenchymal patterns (N1, P1, P2, and DY) that represent a spectrum from a low-density, fatty breast (N1) to a significantly dense breast (P2 and DY).

Breast density varies among individuals according to the inherent relative amounts of fat, connective tissue, and epithelial tissue present *(16)*, all of which can be influenced by endogenous estrogen levels *(17,18)*, the use of postmenopausal hormone replacement therapy (HRT) *(19,20)*, and body mass index *(21)*. A recently published subgroup analysis of the prospective, randomized Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial *(22)* confirmed the results of previous observational studies; i.e., 3 years of estrogen alone or estrogen plus progestin increased mammographic density in 8% and 24% of women, respectively.

In light of the effects of estrogen to increase breast density, the effect of selective estrogen receptor modulators (SERMs) on breast density becomes increasingly important as more SERMs are developed and approved for use in postmenopausal women. SERMs interact with both α and β estrogen receptors, resulting in estrogen agonist or antagonist effects depending on the tissue *(23,24)*. Raloxifene, a SERM with estrogen agonist effects on bone and lipid metabolism and estrogen antagonist effects in the breast and uterus, is approved in the United States for the prevention *(25)* and treatment *(26)* of postmenopausal osteoporosis.

A previous study *(27)* has shown that raloxifene reduces the incidence of invasive breast cancer in postmenopausal women with osteoporosis. However, raloxifene's effects on mammographic breast density have not been studied.

In this study, we evaluated the effects of 2 years of treatment with raloxifene, estrogen, or placebo on breast density using a digitized analysis of mammograms that quantifies the ratio of fibroglandular tissue to fat. We analyzed baseline and

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2-year mammograms from a subgroup of postmenopausal women enrolled in an international osteoporosis prevention trial *(28)*.

Subjects and Methods

Study Design and Subjects

The double-blind, randomized, placebo-controlled osteoporosis prevention trial of 619 postmenopausal women was conducted at 38 study centers in 10 countries in North America, Europe, South Africa, and New Zealand *(28)*.

The osteoporosis prevention trial enrolled healthy postmenopausal women, aged 45 through 60 years, who had undergone hysterectomy no more than 15 years before the study. Women in this trial were to have lumbar spine bone mineral density (BMD) measurements in the range of 2 standard deviations (SDs) above to 2.5 SDs below the mean peak lumbar spine BMD for premenopausal women, inclusive. Women were excluded from the osteoporosis study if they had any of the following: breast cancer at any time or any cancer within the previous 5 years, thromboembolic disorders or cerebrovascular accident, diabetes mellitus, chronic liver disease, impaired kidney function, or clinically significant menopausal symptoms.

Systemic HRT was permitted before, but not during, the osteoporosis study and only if it had been discontinued at least 6 months before study entry. Low-dose vaginal estrogens, except estradiol, were permitted up to three times per week during the study. Concomitant use of progestins, androgens, bone-active agents, or other SERMs was not permitted. Women who had ever used systemic fluoride therapy (except for dental prophylaxis) or had taken bisphosphonates were excluded.

In the osteoporosis trial, a randomized block design was used to assign women to receive either raloxifene HCl (Evista®; Eli Lilly and Company, Indianapolis, IN) at a dose of 60 mg/day or 150 mg/day, conjugated equine estrogens (Premarin®; Wyeth Ayerst, Philadelphia, PA [ERT]) at a dose of 0.625 mg/day, or placebo. Randomization was stratified within each investigative site. Because raloxifene and Premarin pills have different appearances, all women took two pills daily—raloxifene or a placebo of identical appearance and Premarin or a placebo of identical appearance—to maintain the double-blind design of the study. Study visits were scheduled every 3 months for 2 years.

The protocol was approved by local ethical review boards, and all women provided written informed consent for participation in accordance with the principles outlined in the Declaration of Helsinki.

Women were selected for participation in this breast density substudy if they were enrolled at English-speaking sites, had both baseline and 2-year mammograms of the craniocaudal (CC) view that had comparable positioning, and met the osteoporosis prevention study criteria described above (Fig. 1). The analysis of this subset of women was planned before any mammograms were digitized and without prior knowledge of which patients at eligible sites would participate. The "Appendix" section lists the investigators who participated in this breast density substudy.

Breast Density Measurements

Mediolateral oblique (MLO) and CC views of each breast were obtained at baseline and at 2 years. Mammograms taken up to 1 year before study entry were considered to be "baseline." Because the statistical analysis results from the CC and MLO views were similar, all results presented are from the CC view from both breasts. Films were scanned (Lumiscan Model 150; Lumisys, Inc., Sunnyvale CA), and the scanned images were segmented (*see description below*) by the radiologist with a computer-assisted technique called "interactive thresholding." The interactive threshold technique uses software that allows the radiologist to draw a boundary around objects that have similar degrees of whiteness on the

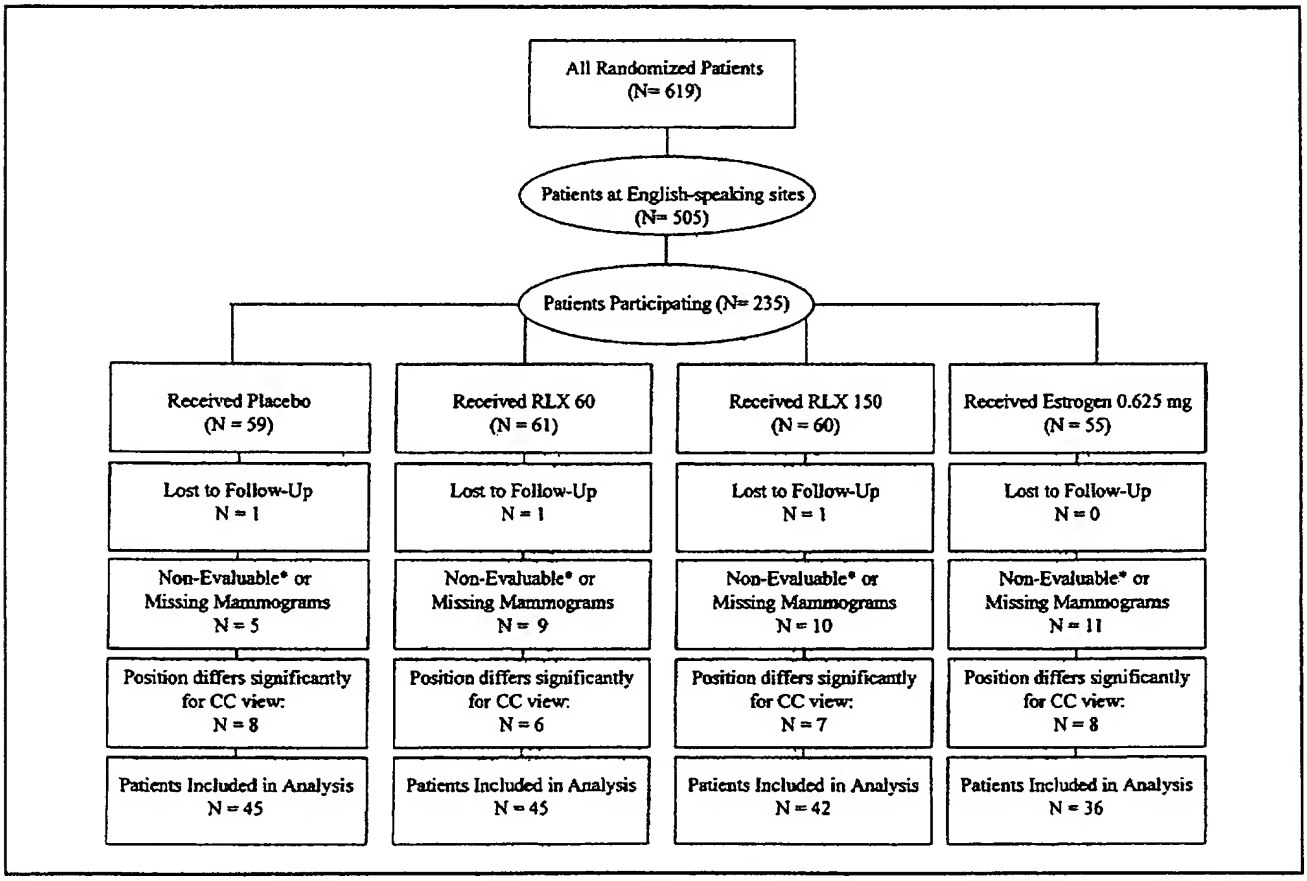

Fig. 1. Patients recruited, enrolled, and analyzed. *Unable to be evaluated because of the presence of breast implants or substantial weight loss. RLX 60 = raloxifene at a dose of 60 mg/day; RLX 150 = raloxifene at a dose of 150 mg/day; CC = craniocaudal.

digitized breast image. To accommodate mammograms with different exposures, a threshold level of whiteness is selected by the radiologist interacting with the computer. Using this method, the radiologist can outline one larger connected area or several separate areas of fibroglandular tissue at the same time in a process called "segmentation." The computer then calculates the area of tissue that has been interactively outlined. The breast region that projects behind the axillary fold was marked and excluded from further analysis.

With the use of this interactive segmentation software developed at Georgetown University Medical Center (Washington, DC), the edge of the breast was first defined by an automated process. One radiologist who was blinded to therapy (M. Freedman), aided by the software, assigned a threshold to the digitized mammograms to define areas of dense breast tissue. A previous study *(29)* has indicated that this method results in very low intra-observer and inter-observer variability. The ratio of the area of dense breast tissue to the total breast area is the percent density.

An example of the results of interactive thresholding is shown in Fig. 2. Fig. 2 represents a 2-year digitized mammogram from one subject (CC view, left breast); the right panel in the figure represents the segmentation of the same mammogram, showing fibroglandular tissue (dark area) and surrounding tissue, as defined by this interactive thresholding method. This example is from a subject assigned to receive raloxifene at a dose of 60 mg/day who had an endpoint density of 7.9%.

Each subject's baseline and 2-year films were viewed together (the temporal sequence of the mammograms was known) to ensure that similar threshold criteria were used, to detect any minor differences in positioning that might limit the accuracy of comparison, to ensure that the films had not been mislabeled, and to detect any artifacts (e.g., silicone breast implants) that might interfere with segmentation. Subjects whose pairs of films showed marked differences in the position of the breast on the radiograph, such that glandular tissue observed on one view could not be observed on the other view, were excluded from further analysis.

Statistical Analysis

Films were analyzed for all subjects who had a baseline and a 2-year mammogram where the positions of both breasts in both mammograms were comparable. For subgroup analyses, continuous demographic variables (age, years since menopause, and body mass index) and baseline density were categorized into tertiles; other subgroup variables included previous HRT use, use of alcohol at entry, and smoking at entry. Subgroup analyses were performed with the use of analysis of variance (ANOVA). Subject demographic variables were analyzed for potential treatment-group differences with the use of a one-way ANOVA for continuous variables and a chi-square test for categorical variables. All statistical tests were two-sided.

The endpoint of this study, change in breast density from baseline to 2 years, was analyzed with the use of ANOVA, with treatment and country as fixed effects in the model. Mammographic density is presented as percent density, calculated as described previously, for both breasts combined. This density definition is a weighted average of the left and right breast densities.

Since breast densities measured by this method have not been described previously, a cutoff value for clinically relevant endpoints had not been determined. We, therefore, chose to use the SD of endpoints in the placebo group in this study to define a potentially clinically relevant endpoint. Any change in breast density greater than the mean change in the placebo group plus 1 SD was considered to be potentially clinically relevant; likewise, any decrease greater than the mean placebo change minus 1 SD was considered to be potentially clinically relevant. This definition of clinical relevance was made before the data had been analyzed and is clearly not the only one that could have been selected. However, this cutoff seemed statistically reasonable, as did the use of the placebo group as an internal reference range.

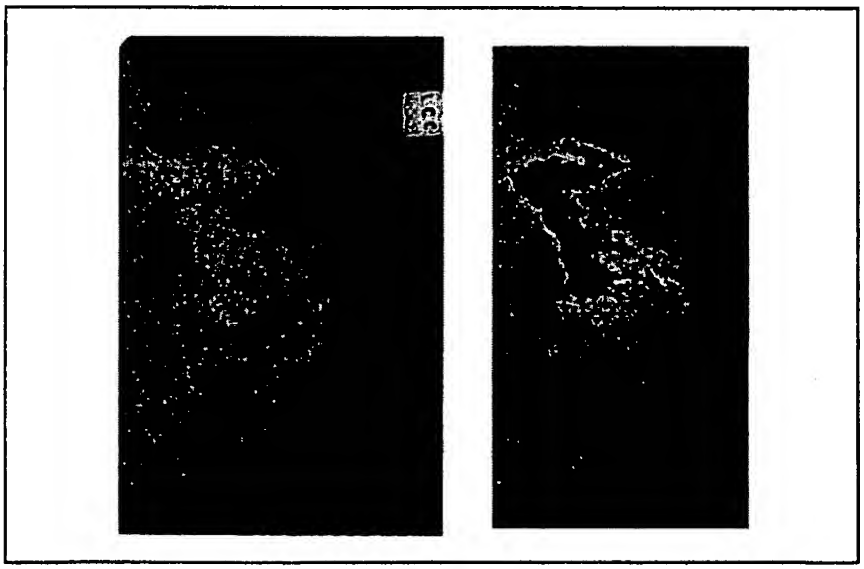

Fig. 2. Paired digitized and segmented mammogram. **Left panel:** digitized mammogram (craniocaudal view, left breast) from a subject treated with raloxifene at a dose of 60 mg/day who had 2-year breast density of 7.9%. **Right panel:** segmentation of the same mammogram, showing fibroglandular tissue (**dark area**) and surrounding tissue, as defined by this interactive thresholding method. Areas of glandular tissue intermixed with fat appear as **areas of spotty or irregular intermixed black and gray.**

Results

Of the 235 participating patients, 38 were lost to follow-up or had mammograms that were missing or had substantial differences in breast position between baseline and 2 years. Of the remaining 197 women with paired mammograms with comparable positioning, 168 had CC mammograms from both breasts that showed no substantial differences in position between the baseline and the 2-year films (Fig. 1). Data from these 168 patients are presented here. The mean age and years after menopause were 53 years and 6 years, respectively; additional patient characteristics are presented in Table 1. There were no statistically significant therapy-group differences for any patient characteristic at baseline, and the characteristics of the subset of women enrolled in this study were not different from those of the other 451 subjects enrolled in the overall osteoporosis prevention trial (data not shown).

After 2 years of treatment, the change in mean breast density in the ERT group (+1.2%) was statistically significantly different from that in each of the other groups (−1.3%, −1.5%, and −1.7% for the groups receiving placebo, raloxifene at a dose of 60 mg/day, and raloxifene at a dose of 150 mg/day, respectively; $P<.01$ for all three comparisons). The mean density within the placebo group and both raloxifene groups decreased statistically significantly from baseline ($P<.02$ for each group), while the mean density in the ERT group increased but not statistically significantly (Table 2). Among all of the variables tested (age, years since menopause, body mass index, previous HRT use, use of alcohol at entry, smoking at entry, and baseline breast density), there were no statistically significant therapy-by-subgroup interactions.

Table 3 lists the percentage of patients in each group who experienced a change in breast density greater than 1 SD above or below the mean change observed in the placebo group; these changes were considered to be "potentially clinically relevant." Potentially clinically relevant changes, according to our definition, were observed for 11.1% of women on placebo, 6.7% of women on raloxifene at a

Table 1. Baseline characteristics of study subjects*

Characteristic	Treatment group			
		Raloxifene		
	Placebo (n = 45)	60 mg/day (n = 45)	150 mg/day (n = 42)	Estrogen, 0.625 mg/day (n = 36)
Age, y†	52.2 ± 5.1	54.1 ± 4.2	53.3 ± 4.8	52.1 ± 4.6
Years since menopause†	5.9 ± 4.1	5.5 ± 3.9	6.6 ± 4.1	6.0 ± 4.5
Body mass index, kg/m^2†	28.7 ± 4.7	27.1 ± 4.8	28.1 ± 4.6	27.0 ± 5.2
Previous HRT use, yes	33%	27%	33%	36%
Use of alcohol at entry‡	22%	27%	24%	33%
Smoking at entry, yes	13%	24%	17%	17%
Baseline breast density, CC view†	9.8% ± 9.6%	9.3% ± 9.1%	8.1% ± 6.6%	13.5% ± 11.5%

*There were no statistically significant therapy-group differences for any baseline patient characteristic, and the subset of women enrolled in this study was not different from the rest of the subjects enrolled in the larger trial (data not shown). kg/m^2 = (weight in kilograms)/(height in meters)^2; HRT = hormone replacement therapy; CC = craniocaudal.

†Data are presented as mean ± standard deviation.

‡Patients' self-reported consumption of more than three alcoholic drinks per week.

Table 2. Breast density at baseline and after 2 years by treatment group*

Breast density	Treatment group			
		Raloxifene		
	Placebo (n = 45)	60 mg/day (n = 45)	150 mg/day (n = 42)	Estrogen, 0.625 mg/day (n = 36)
Baseline	9.8% ± 9.6%	9.3% ± 9.1%	8.1% ± 6.6%	13.5% ± 11.5%
2 y	8.5% ± 11.4%	7.7% ± 8.8%	6.4% ± 5.3%	14.6% ± 11.4%
Mean change	−1.3% ± 2.9%	−1.5% ± 4.1%	−1.7% ± 3.4%	+1.2% ± 5.3%†
95% confidence interval‡	−2.2 to −0.4	−2.7 to −0.3	−2.8 to −0.6	−0.6 to 3.0
P	.003	.002	<.001	.611

*Data are presented as mean ± standard deviation for craniocaudal views. *P* values are based on Student's *t* test.

†At 2 years, the mean change in breast density in the estrogen group was statistically significantly greater than that in each of the other three groups ($P<.01$ for all three comparisons).

‡95% confidence intervals for within-group mean changes.

Table 3. Percentage of patients with a change in breast density beyond the 1-standard-deviation threshold observed in the placebo group at 2 years*

Breast density change	Treatment group, %			
		Raloxifene		
	Placebo (n = 45)	60 mg/day (n = 45)	150 mg/day (n = 42)	Estrogen, 0.625 mg/day (n = 36)
Increase >1 SD above the mean placebo change	11.1	6.7	9.5	30.6
Within 1 SD of mean placebo change	71.1	80.0	71.4	58.3
Decrease >1 SD below the mean placebo change	17.8	13.3	19.0	11.1

*Changes in breast density were determined for craniocaudal view mammograms. Changes greater than 1 standard deviation (SD) above or below the mean change observed in the placebo group were considered to be potentially clinically relevant. There was a significant therapy difference for the proportion of women whose breast density increased beyond the 1-SD threshold (1.6%) versus those who did not (two-sided chi-square, $P = .010$). The therapy difference was not significant for the proportion of women whose breast density decreased beyond the 1-SD threshold (4.2%) versus those who did not (two-sided chi-square, $P = .735$).

dose of 60 mg/day, 9.5% of women on raloxifene at a dose of 150 mg/day, and 30.6% of women on ERT (chi-square; $P = .010$).

Most (98%) of the patients in this substudy reported at least one adverse event during the first 24 months of the trial. Breast pain was reported by 18 patients (10.7%), of which four were assigned to placebo, two were assigned to raloxifene at 60 mg/day, four were assigned to raloxifene at 150 mg/day, and eight were assigned to ERT. (The therapy-group differences were not statistically significant for the proportion of patients reporting this adverse event.) No cases of breast cancer were reported for women participating in this breast density substudy.

Discussion

In this subset of 168 postmenopausal women enrolled in an international osteoporosis prevention trial, the mean breast density decreased by 1.3% in the group receiving placebo, by 1.5% in the group receiving raloxifene at a dose of 60 mg/day, and by 1.7% in the group receiving raloxifene at a dose of 150 mg/day, but it increased in the ERT-treated group by 1.2% at 2 years. The decreases in mean breast density observed with placebo and raloxifene may be attributed partially to age-related decreases in breast density *(30)*. In an analysis of changes either 1 SD above or 1 SD below the mean change in breast density observed in the placebo group, which we considered to be potentially clinically relevant, twice as many women assigned to receive raloxifene had decreases in breast density below the lower 1-SD threshold (13.3% and 19.0% for the groups receiving 60 mg/day and 150 mg/day, respectively) as had increases above the upper 1-SD threshold (6.7% and 9.5%, respectively). In contrast, three times as many women assigned to ERT had a mean change in breast density that was an increase above the upper 1-SD threshold (30.6%) as had a decrease below the lower 1-SD threshold (11.1%).

It has been reported previously that treatment with raloxifene, in addition to its effects on bone density, results in a 76% reduction in the incidence of invasive breast cancer in postmenopausal women with osteoporosis by 40 months of therapy *(26,27)*. This is consistent with the estrogen-antagonist effect of raloxifene in the breast, as is the lack of an effect of raloxifene on breast density observed in the current study. Because raloxifene does not increase breast density, such treatment should also not impede the detection of new breast cancer by mammography.

The absolute mean breast densities measured in this trial were smaller than those observed in another study *(22)* with

similar study populations, a difference that is due, in part, to the various methods used for measuring breast density. In the most commonly used method, radiologists visually inspect mammograms and assign them to one of four subjective categories of breast density *(6,8,18,31)*. Other methods estimate either the percentage of parenchymal tissue present *(32)* or the changes in density by comparing mammograms taken at different times *(19,22,33)*. All of these methods are visual, subjective, and qualitative. In contrast, quantitative methods that exclude interspersed fat from the analysis of glandular tissue by manual segmentation *(6,8,31)* or interactive computer analysis *(4,34)*, as was done in this study, measure only glandular tissue and result in overall lower breast density values.

In the recently published PEPI trial *(22)*, 8% of the patients on ERT and 20%–24% of those on HRT (estrogen plus progesterone) had increases in breast density at 12 months. These women had to have at least a "moderate" change in breast density for a change to be recognized in the four-category system used to analyze data in that study. Compared with the women in our trial, PEPI trial participants were about 6 years older, half as likely to have ever used HRT, and were not required to have undergone hysterectomy. Compared with the ERT group in the PEPI trial, a higher percentage (30.6%) of the ERT group in our trial demonstrated a potentially clinically relevant change. This difference is most likely due to the increased sensitivity of the method employed in our study. Despite the differences in density evaluation methods and populations, statistically significantly more patients receiving ERT in both studies developed increased breast density compared with those receiving placebo. The effects of other SERMs, such as tamoxifen or toremifene, on breast density have not been reported in healthy postmenopausal women without breast cancer.

As new therapies, such as SERMs, become available, it is desirable to understand whether specific characteristics predict enhanced response to therapy. For example, some subgroups of women (e.g., those with a higher percentage of glandular tissue) might be more responsive to estrogen or raloxifene, since both therapies work through estrogen receptors that are present in glandular tissue. However, while this study was not sufficiently powered to detect such differences, a trend in that direction was observed.

This study has two primary limitations. First, because this trial used ERT instead of HRT (estrogen plus progesterone), we could not discern the effects of progestin on breast density separately from the effects of estrogen. In other trials *(20,22)*, estrogen plus progestin (given cyclically or in combination) increased breast density beyond the increase observed for estrogen alone. Second, although the digitizing method used here is similar to one published previously *(3)*, to our knowledge, this is the first clinical trial application of this particular method. We are, therefore, unable to directly compare our results with the results of other studies.

Thus, in a study of healthy postmenopausal women enrolled in an international osteoporosis prevention trial, raloxifene given at a dose of 60 mg/day or 150 mg/day did not increase breast density at 2 years. These findings suggest that raloxifene therapy should not impair detection of new breast cancer by mammography. The lack of apparent mammary parenchymal stimulation by raloxifene clearly distinguishes its effects on breast tissue from those of conjugated estrogens, which reproducibly increase breast density. These different effects on breast density may be of benefit in evaluating screening mammograms for women who are receiving raloxifene or HRT to prevent or treat osteoporosis.

APPENDIX

The following investigators participated in this breast density substudy: **Australia**—J. A. Eisman, Bone and Mineral Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney; A. G. Need, Department of Clinical Biochemistry, Institute of Medical and Veterinary Science, Adelaide; K. W. Ng, Department of Medicine, Saint Vincent's Institute of Medical Research, Fitzroy; R. L. Prince, Department of Medicine, Sir Charles Gairdner Hospital, Nedlands; and E. Seeman, Department of Endocrinology, The Austin Hospital, Heidelberg. **Canada**—J. D. Adachi, St. Joseph's Hospital, Hamilton; J. P. Brown, Le Centre Hospitalier de L'Universite Laval, Sainte-Foy; D. A. Hanley, Division of Endocrinology and Metabolism, University of Calgary; A. B. Hodsman, Saint Joseph's Health Center, London; R. G. Josse, Division of Endocrinology and Metabolism, Saint Michael's Hospital, Toronto; W. P. Olszynski, Midtown Medical Centre, Saskatoon; L. G. Ste-Marie, Hospital St. Luc, Viallet, Montreal; and J. P. Wade, The Arthritis Centre, Vancouver. **United Kingdom**—R. Eastall, Northern General Hospital, University of Sheffield; I. Fogelman, Osteoporosis Department, Guys Hospital, London; and I. Smith and S. Young, Coppull Menopause and Osteoporosis Research Unit, Lancashire. **New Zealand**—I. R. Reid, University of Auckland School of Medicine. **South Africa**—B. H. Ascott-Evans, Department of Nuclear Medicine, Groote Schuur Hospital, Cape Town; S. Hough, Department of Endocrinology and Metabolism, Tygerberg Hospital; and C. M. Schnitzler, Department of Orthopaedic Surgery, University of the Witwaterstrand, Parktown. **United States**—J. F. Aloia, Winthrop-University Hospital, Mineola, NY; H. G. Bone III, St. John Hospital Bone and Mineral Clinic, Detroit, MI; C. Cheney, Scripps Clinical Research Foundation, La Jolla, CA; M. H. Davidson, Chicago Center for Clinical Research, IL; S. Greenspan, Beth Israel Hospital, Boston, MA; A. A. Licata, Department of Endocrinology, Osteoporosis/Metabolic Bone Disease Clinic, Cleveland, OH; M. R. McClung, Center for Osteoporosis Research, Portland, OR; M. T. McDermott, Austin Diagnostic Clinic, Center for Clinical Research, TX; P. D. Miller, Colorado Center for Bone Research, Lakewood; R. A. Sachson, Research Institute of Dallas, TX; and N. B. Watts, Division of Endocrinology, Emory Clinic, Atlanta, GA.

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NOTES

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Mammographic changes associated with raloxifene and tibolone therapy in postmenopausal women: a prospective study.

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OBJECTIVE: The prolonged use of estrogen therapy is associated with a slightly increased risk of breast cancer. Alternative therapies that are effective in the prevention of menopause, having associated morbidities but no unwanted effects, are of primary interest in the pharmacologic research. The aim of this study was to evaluate the effect of two alternative to estrogens drugs, the selective estrogen receptor modulator raloxifene and the tissue-specific tibolone, on the mammographic appearance of the breast. DESIGN: The study group comprised 131 postmenopausal women aged 41 to 67 years. The women were at least 2 years postmenopausal, free of climacteric symptoms, and at the time of entry to the study had not had therapy for at least 9 months. Women with risk factors for osteoporosis or cardiovascular disease were allocated either to tibolone (n = 56) or raloxifene (n = 48) therapy. Women with no risk factors and women who either did not qualify for or denied treatment (n = 27) served as controls. The study duration was 12 months. Women received a baseline mammogram before commencing therapy and a repeat mammogram at the end of the study period. Mammogram findings were classified according to the modified Wolfe criteria by two expert radiologists. RESULTS: No difference was identified between groups with respect to baseline characteristics associated with breast cancer risk. Similarly, no difference was detected between groups concerning the modified Wolfe classification of baseline mammographic findings. In the tibolone group, 10.7% of the women showed an increase in

breast density in the 12-month reevaluation. The respective figure in the raloxifene group was 6.3%, whereas no woman in the control group showed an increase in breast density. Differences in the increase in breast density between groups did not, however, reach statistical significance. Accordingly, 10.7% of women in the tibolone group and 18.8% of women in the raloxifene group exhibited involutionary changes in the repeat mammogram, whereas 25.9% of women in the control group revealed a decrease in breast density in the 12-month examination. The percentages were not significantly different between groups. CONCLUSIONS: Breast density as shown by mammography was stable in a majority of patients and changed in a minority of cases for both tibolone and raloxifene. In most patients, these drugs are not likely to interfere with mammogram interpretation. Larger long-term studies are needed to confirm the impact of prolonged tibolone or raloxifene administration on mammography.

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